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*ON THE MAGNETIC ASYMMETRY OF MUSCLE FIBERS**

By William Arnold, Richard Steele and Helmut Mueller

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AND
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Communicated by Albert Szent-Györgyi

On November 4, 1845, Faraday discovered diamagnetism. On December 18 he read a paper[1] before the Royal Society on this "New Magnetic Action." At that time he had already shown that his magnet repelled a number of biological specimens, such as wood, ivory, dried mutton, fresh and dried beef, fresh and dried blood, leather, and apple. Since Faraday's original research, there have been surprisingly few studies on the magnetic properties of biological objects. On the subject of magnetic anisotropy we have found only four papers; three are by Nilakantan[2] on mother of pearl, molluscan shells, and teakwood, respectively; the fourth is by Loeb and Welo[3] on cellulosic materials.

Our interest in this subject grew out of an observation made in Dr. Szent-Györgyi's laboratory in the summer of 1956. A piece of muscle fiber, several times longer than it was wide, was suspended in an inhomogeneous magnetic field; it tended to set its long axis at right angles to the direction of the field, as was to be expected of a diamagnetic object. A shorter piece of muscle fiber, whose length was one and one-half times its width, was found to set its long axis parallel to the field. This curious behavior could be understood if the muscle fiber were anisotropic in its magnetic properties. It is our purpose in this paper to show that this is so.

An object with no permanent magnetic moment, when suspended in a horizontal magnetic field, is acted on by four different torques around the vertical axis.

1. Assume the sample to be anisotropic, with K_1 as the volume susceptibility in one horizontal direction and K_2 in the other; K_1 is taken in the direction in which the algebraic value of the susceptibility is greatest. Then, if V is the volume of the sample, H the strength of the field, and θ the angle between K_1 and the direction of the field, there is a torque given by

$$V(K_1 - K_2)H^2 \cos\theta \sin\theta$$

tending to turn the object so as to make K_1 parallel to the field.

2. Unless the object is a sphere, it will have different demagnetizing factors in the different directions. This effect gives a torque tending to set the long axis of the sample parallel to the field. However, since the magnitude of this torque

depends upon the square of the susceptibility, it will be negligible. In fact, this torque has never been detected for any diamagnetic object.

3. The poles of the sample will form magnetic images in the iron of the pole pieces, but again the torque depends upon the square of the susceptibility and thus can be neglected except for ferromagnetic samples.

4. Unless the magnetic field is homogeneous, the forces that tend to move diamagnetic material from regions of strong field to regions of weak field can produce a torque on any nonspherical sample. This is the torque observed by Faraday and the one commonly seen when a diamagnetic sample is suspended between the poles of a magnet.

From the above we see that the only sensible torque acting on a diamagnetic sample suspended in a uniform magnetic field is the one due to magnetic anisotropy. This is essentially the method developed and used by Krishnon.[4]

Mr. Harlow, of the Marine Biological Laboratory, constructed for us a large magnet that has a very uniform field. The pole pieces are soft steel 3×3 inches; when they are separated by 5/8 inch, the field is about 3,000 gauss. The magnetomotive force is furnished by two large Alnico magnets.

In order to show that the magnetic field was sufficiently homogeneous, we suspended in it two 1/8-inch diameter glass balls, cemented together so as to form a figure 8-shaped object when looked at from above. The suspension was a fine nylon filament some 63 cm. long. The upper end of the suspension was fastened to a torsion head that could be rotated about the vertical axis. The angle of rotation we shall call ψ. Curve A gives the angular position of the two balls as the torsion head was rotated; as can be seen, the curve is fairly straight. It must be remembered that, since glass is more diamagnetic than muscle and the two balls are larger than the muscle samples that we used, the torque due to any inhomogeneity of the magnetic field is several times larger in the case of the glass balls than for the muscle sample (see Fig. 1).

A sample of fresh rabbit psoas muscle, $6 \times 3 \times 3$ mm., was used to give curve B. The curve is discontinuous because of the anisotropy of the muscle. As the torsion head is rotated, the sample rotates in the same sense but through a smaller angle until θ reaches a critical value somewhere between 45° and 90°, whereupon the sample suddenly spins to a new position of equilibrium. If we let A be the torsion constant of the suspension, the total torque (T) on the sample can be written as

$$T = A(\psi - \theta) - V(K_1 - K_2)H^2 \cos\theta \sin\theta = 0,$$

where the first term is due to the suspending filament and the second term is due to the magnetic anisotropy. Both ψ, the angle of the torsion head, and θ, the angle of the sample, are measured from the stable equilibrium position in which K_1 is parallel to the field. Experimentally, K_1 is found to be in the direction of the muscle fiber. For the sample to be stable at any angle θ we must have $dT/d\theta$ negative:

$$\frac{dT}{d\theta} = -A - V(K_1 - K_2)H^2 (\cos^2\theta - \sin^2\theta)$$

Thus, whenever

$$A < V(K_1 - K_2)H^2,$$

we shall have a critical angle at which the sample becomes unstable and spins. This spinning is the most dramatic evidence of magnetic anisotropy. If the sample is large and the torsion constant sufficiently small, the spin may amount to four or five complete revolutions of the sample.

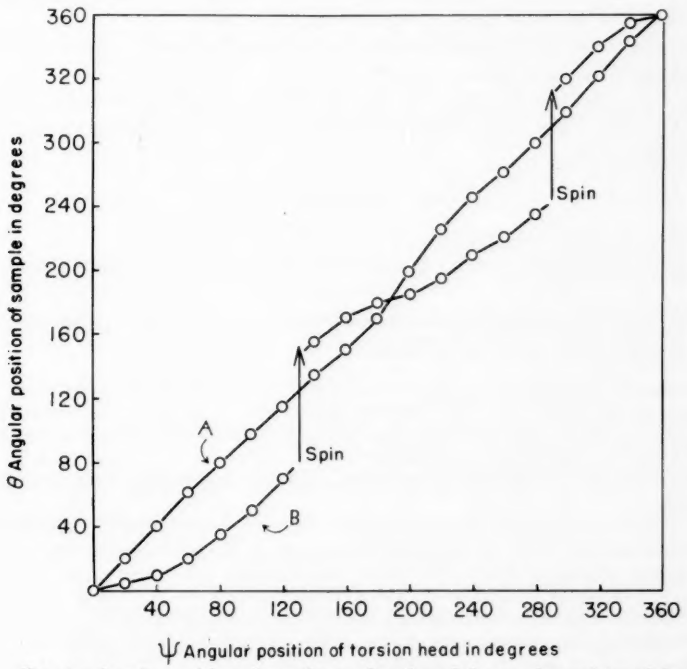

FIG. 1.—Angular position of sample as a function of the angular position of the torsion head. Curve *A* is for the two glass balls. Curve *B* is for a sample of psoas muscle.

The apparatus available does not allow us to make precise quantitative measurements; however, a rough estimate of the torsion constant and critical angle shows that $(K_1 - K_2)$ is approximately 2×10^{-8} c.g.s. units/cc. We do not know the magnetic susceptibility of muscle fiber, but the observation that, when suspended in air, muscle fiber is repelled by an inhomogeneous magnetic field shows that it is diamagnetic, just as Faraday reported. The observation that, when suspended in water, the fiber is attracted by a magnet shows that it is less diamagnetic than water. The magnetic susceptibility of water is -0.7×10^{-6}. The asymmetry found for muscle is as large as that observed for many crystals.

Although determination of magnetic properties of muscle before and after contraction would certainly be of great interest, our apparatus again does not permit precise quantitative measurements. Experiments show that in fresh muscle, contracted by freezing and thawing, the asymmetry has the same sign in both states. However, in the case of freeze-dried muscle, the asymmetry changes sign upon contraction brought about by dipping the preparation in water.

A sample of muscle fiber, suspended in air as we have described, can be observed for many hours. If the sample is allowed to dry slowly, it becomes less and less diamagnetic as the drying proceeds. Eventually, many of our samples became paramagnetic. During the drying, the magnetic anisotropy remains; this means that at a certain stage in the process the muscle fiber is paramagnetic in the direction of the fiber axis and diamagnetic normal to that axis.

Glycerated[5] muscle preparations, as well as air-dried and freeze-dried muscle fibers, showed strong magnetic anisotropy.

Both nerve and tendon taken from the rabbit showed asymmetric magnetic properties. In a sample of fresh beef liver no magnetic anisotropy could be detected with certainty.

SUMMARY

1. The magnetic susceptibility of muscle fiber is asymmetric.
2. The muscle fiber is more diamagnetic normal to its long axis than parallel to it.
3. We feel that magnetic measurements can be made to give useful information about the structure of biological tissues.

* This research was sponsored by the grant H-2042R of the National Heart Institute, a grant from the Commonwealth Fund, the Muscular Dystrophy Associations, the National Science Foundation, the American Heart Association, the Association for the Aid of Crippled Children, and the United Cerebral Palsy Associations.

Dr. Mueller's work was done during the tenure of a Research Fellowship of the American Heart Association.

The writers much appreciate Dr. Albert Szent-Györgyi's continued interest in this problem. Dr. Arnold is grateful to Drs. Weinberg and Hollaender, of the Oak Ridge National Laboratory, for permission to work in Woods Hole during the summers. Dr. Steele's new address is the Biochemistry Department, Tulane University Medical School.

[1] M. Faraday, *Experimental Researches in Electricity* (London, 1855), **3,** 27.

[2] P. Nilakantan, *Proc. Indian Acad. Sci.*, A, **2,** 621, 1935; **4,** 542, 1936; **7,** 38, 1938.

[3] L. Loeb and L. A. Welo, *J. Textile Research*, **23,** 251, 1953.

[4] K. S. Krishnon, B. C. Guha, and S. Banerjee, *Phil. Trans. Roy. Soc. London*, A, **231,** 235, 1933.

[5] A. Szent-Györgyi, *Biol. Bull.*, **96,** No. 2, 144, 1949.

Since writing the above we have found a fine paper by E. Cotton-Feytis and Emmanuel Fauré-Frémiet, *Compt. rend.* **214,** 996–998, 1942 in which they made magnetic measurements on silk, hair, horn and tendon.

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*MOLECULAR GROWTH REQUIREMENTS OF SINGLE MAMMALIAN CELLS: THE ACTION OF FETUIN IN PROMOTING CELL ATTACHMENT TO GLASS**

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Communicated by Dr. George Gamow, October 23, 1957

In an earlier paper[1] it was shown that the growth requirements for small molecules exhibited by single S3 HeLa cells varied with the degree of dialysis of the

macromolecular fraction of serum used as a growth supplement[2] and a medium was defined which permitted good colony formation from single cells, even with extensively dialyzed serum protein preparations. Other experiments showed (*a*) that growth of S3 HeLa cells occurs only in media which render the cell capable of attachment and stretching when a nonagitated suspension is placed on a clean glass surface;[3] (*b*) that serum from different mammalian species[4] and from human subjects of different age groups produces markedly different degrees of stretching of glass-attached cells; and (*c*) that much, if not all, of this glass-attachment and stretching activity is associated with the macromolecular serum component. Fractionation of the macromolecular serum supplement revealed that at least two protein constituents play active roles in cell growth in vitro: serum albumin, which may function at least partly as a carrier of essential small molecules, and an α-globulin, which is important in the attachment of the cells to the glass surface.[1]

After these experiments were completed, a communication by Lieberman and Ove appeared[5] in which the cell-stretching activity of an α-globulin-containing fraction of normal serum was described, with properties similar to that of our preparation. In this paper we describe our experiments dealing with the isolation of an α-globulin-containing fraction from adult serum which promotes attachment and stretching of cells on a glass surface. Further experiments are presented, demonstrating that similar activity is possessed by the Fetuin system of fetal serum and that both Fetuin and the material isolated from normal serum exercise a strong antitryptic action, which may contribute to their biological effectiveness in promoting cell growth in vitro.

METHODS AND MATERIALS

The cells employed were the S3 clonal strain of HeLa, previously described.[4] Cells were trypsinized by our standard technique, plated in the desired number in Petri dishes containing test medium, and incubated at 38° C. in an atmosphere of 5 per cent CO_2. Attachment to glass was measured by microscopic counting on the glass surface either of viable cells or of fixed and stained preparations, a technique also utilized independently by Lieberman and Ove. The titers of the "cell-stretching factor" obtained by us are meaningful only under the specific experimental conditions employed, since the apparent activity of a solution varies greatly with the time of cell incubation and other factors.

EXPERIMENTAL RESULTS

Trypsinized S3 cells pipetted onto a clean glass surface in the presence of normal human, bovine, or equine sera become attached to the glass within 2 hours at 38° C. and stretch out over an area of about 1,000 μ^2.[4] Good attachment and stretching, as illustrated in the photographs of Figure 1*A*, *B*, *C*, are obtained even with serum diluted to about 2 per cent of its original concentration. In the absence of an adequate concentration of the proper serum fraction, the cells either fail to attach or else become loosely and temporarily fixed to the glass as spheres. Such glass attachment and stretching do not occur with the micromolecular serum constituents alone but readily take place with the dialyzed macromolecular portion of serum resuspended in Hanks saline. Fractionation of the active, undialyzable material by

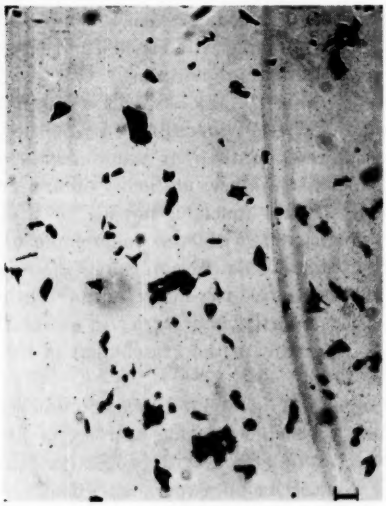

FIG. 1A. Photomicrograph demonstrating the stretched condition of the cells in an adequate concentration of purified Fetuin.

FIG. 1B. Photomicrograph demonstrating the rounded condition of cells, which will shortly be released from their bond to the glass in the absence of any "stretching factor."

FIG. 1C. Photomicrograph demonstrating the stretched condition of the cells in an adequate concentration of normal serum. The black line represents a distance of 100 μ.

means of $(NH_4)_2SO_4$ was carried out as shown in Table 1, and the activity was found to follow the α-globulin fraction, precipitating in 35–50 per cent saturation of $(NH_4)_2SO_4$. Albumin and γ-globulin displayed no cell-stretching activity. In most of the following experiments, 5–10 mg/cc of purified albumin[6] were added to the medium, because it appeared to assist the nutritional state of the cells with-

out affecting appreciably their response to the "stretching factor." The active fraction was repeatedly precipitated with 45.0 per cent saturated $(NH_4)_2SO_4$ solution, resulting in an α_1-globulin-rich material, of which 70 μg/cc were sufficient to cause dispersed cells to attach to and stretch out on a glass surface and remain in this condition for some days.

TABLE 1

FRACTIONATION PROCEDURE EMPLOYED FOR THE α-GLOBULIN FRACTION OF NORMAL (AND FETAL) SERA

A. One-half volume of saturated $(NH_4)_2SO_4$ is added to 1 volume of serum and allowed to stand several hours at 5° C. and neutral pH. The precipitate is removed and discarded or may be further fractionated to yield purified γ-globulin.
B. The supernatant is adjusted to 50 per cent saturation of $(NH_4)_2SO_4$ by adding the proper volume of saturated $(NH_4)_2SO_4$ and again allowed to stand as described above. The supernatant is discarded.
C. The precipitate is resuspended in 0.85 per cent saline and dialyzed exhaustively against running tap water and finally against 0.85 per cent saline.
D. Steps A through C are repeated two more times as above.
E. Two final cycles of fractional precipitation by $(NH_4)_2SO_4$ are employed, wherein the fraction soluble at 40 per cent saturation and insoluble at 45 per cent saturation of $(NH_4)_2SO_4$ is recovered.
F. The final protein solution is filtered as a concentrated solution through a 0.02 porosity Selas filter and then its protein content determined by the quantitative Biuret reaction.

The properties of this protein material were reminiscent of Fetuin, the α_1-globulin component which comprises 45 per cent of calf fetal serum protein.[7] Therefore, study was initiated to determine whether fetal serum contains more cell-stretching factor than that of more mature animals. Sera were collected from normal and from fetal calves and titrated for their content of stretching factor by noting the highest dilution which would cause attachment and stretching of an S3 cell suspension under standard conditions. Typical results, shown in Table 2, demonstrated that the fetal calf serum contains approximately 10 times as much activity as that from the normal calf.

TABLE 2*

COMPARISON OF THE CONTENT OF STRETCHING FACTOR IN POOLED NORMAL AND FETAL CALF SERA

SERUM CONCENTRATION (Per Cent)	PER CENT OF ORIGINAL CELL INOCULUM REMAINING GLASS-ATTACHED AND STRETCHED AFTER 18 HOURS AT 37° C.	
	Normal Calf	Fetal Calf
10	66.8	105.0
3	34.2	105.0
1	0.3	107.0
0.3	0.0	35.2
0.1	0.0	1.0
0.03	0.0	0.0

* 5×10^4 cells of a monodisperse S3 suspension were pipetted into Petri dishes in a medium containing the indicated amount of serum, 40 per cent of the standard nutrient solution previously described, and Hanks saline to 100 per cent. The plates were fixed and stained after 18 hours of incubation at 38° C., and the number of glass attached, stretched cells on each plate was calculated from microscopic counts of 10 or more representative fields.

Calf fetal serum was fractionated in accordance with the procedure described by Pedersen for isolation of Fetuin.[7] The resulting preparation revealed a single electrophoretic component at pH 8.6, with a mobility of -4.4×10^{-5} cm^2 volt^{-1} sec^{-1} (Fig. 2*A*). Ultracentrifugal analysis resulted in a pattern in which 95 per cent of the material moves with a single boundary, with a sedimentation constant of 2.9 Svedberg units (Fig. 2*B*). Test with diphenylamine reagent[7] indicated the

FIG. 2A. A descending electrophoretic pattern of purified Fetuin. The protein concentration is 1.1 per cent in diethylbarbiturate buffer, $\Gamma/2 = 0.1$, at pH 8.6. The slight shoulder indicates some heterogeneity. At pH 4.0 in acetate buffer and the same ionic strength, the material is about 95 per cent homogeneous electrophoretically with mobility indicated in Table III.

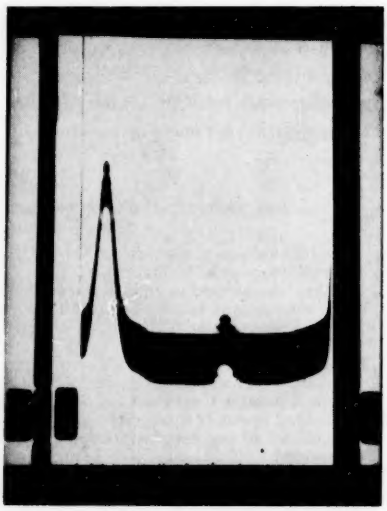

FIG. 2B. A sedimentation pattern of purified Fetuin. The protein concentration and buffer are as in Fig. 2A. The large peak at the left is the slower moving peak corresponding to a sedimentation constant of 2.9 Svedberg units, while the smaller one represents the material with a constant of 17.6 Svedberg units.

presence of sialic acid. These and other properties of the Fetuin prepared in the course of these experiments are summarized in Table 3.

TABLE 3

COMPARISON OF PROPERTIES OF OUR FETUIN PREPARATION WITH THOSE DESCRIBED BY PEDERSEN AND BY DEUTSCH*

Properties	Our Fetuin Preparation	Fetuin Prepared by Pedersen and by Deutsch
Water solubility	Soluble	Unknown
Per cent $(NH_4)_2SO_4$ saturation at which precipitation is initiated	38	38
Solubility in 5 per cent trichloroacetic acid (TCA)	Soluble	Soluble
Electrophoretic mobility ($cm^2\ volt^{-1}\ sec^{-1}$)		
pH 8.6	-4.4×10^{-5}	
pH 4.0	-0.9×10^{-5}	-0.9×10^{-5} (major component)
Sedimentation constant	95 per cent: 2.9 Svedberg units 5 per cent: 17.6 Svedberg units	96 per cent: 2.86 Svedberg units 3–4 per cent: 18.0 Svedberg units
Diphenylamine test for sialic acid of mucoprotein	Positive	Positive

* K. O. Pedersen, *J. Phys. and Colloid Chem.*, **51**, 164, 1947; H. F. Deutsch, *J. Biol. Chem.*, **208**, 669, 1954.

Our Fetuin preparations are highly potent in promoting cell attachment and stretching on glass, equaling in specific activity the most active fractions prepared from the serum of normal animals or adult humans. The total yield of the "stretching factor" from fetal serum is much higher than that obtained from normal adult

serum. This attachment activity of Fetuin requires the presence of divalent cations and can be prevented completely by 10^{-3} M versene. Mg in a concentration of 3×10^{-4} M is maximally active as the stretching cofactor. Ca is less active, while Zn, Cd, Co, and Cu are ineffective.

Fetuin is active as an inhibitor of proteolysis by agents like trypsin.[7] Since mammalian cells are known to be strongly proteolytic when grown in vitro,[8] the mechanism by which Fetuin promotes cell attachment and stretching on a glass surface might involve inhibition of the cellular tryptic action. Hence experiments were designed to test whether Fetuin can prevent the liberation of glass-attached cells by trypsin. Petri dishes were seeded with 2×10^4 S3 cells in complete nutrient medium[4] and incubated at 37° C. in 5 per cent CO_2 for 3–18 hours to insure excellent cell attachment and stretching on the glass. The medium was then removed, the cells washed three times in Hanks saline minus calcium and magnesium, and then resuspended in a trypsin-saline mixture, together with various concentrations of different serum fractions. The results are shown in Table 4 and demonstrate that both Fetuin and the less pure α_1-globulin isolated from adult serum are highly effective in preventing tryptic release of glass-attached cells, in concentrations at which albumin is without activity. Similar experiments showed that γ-globulin also is inactive.

TABLE 4

DEMONSTRATION OF ABILITY OF FETUIN AND OF LESS PURE α_1-GLOBULIN FROM ADULT HORSE SERUM BUT NOT OF ALBUMIN TO PREVENT DETACHMENT OF CELLS FROM GLASS SURFACE BY TRYPSIN*

PROTEIN CONCENTRATION (MG/ML)	PROTEIN TESTED		
	FETUIN	α_1-Globulin from Adult Normal Horse	Human Albumin
2.0	115.%	172.%	37.%
0.4	58.%	4.%	0
0.1	1.%	0.%	0

* Each Petri dish received 5×10^4 S3 cells in complete growth medium containing 30 per cent whole serum. After incubation for several hours, during which all the cells become attached to the glass surface and highly stretched, the liquid was removed, the cells washed three times in saline lacking divalent cations, and 5.0 cc. of this saline, plus the indicated concentrations of proteins, were added. Then trypsin in a final concentration of 0.01 per cent was added to each plate, and all plates were incubated at 38° C. in 5 per cent CO_2 for 10 minutes, after which the number of cells remaining attached in each plate was determined by microscopic counting. The figures above indicate the percentage of cells which remain stretched on glass relative to a control without trypsin.

These experiments do not establish whether the antitryptic factor associated with the α-globulin of normal serum is identical with the much more abundant "stretching factor" present in the Fetuin fraction of fetal serum. The material isolated from normal serum by Lieberman and Ove exhibits two electrophoretic components at pH 8.6 and a pair of sedimentation boundaries, of which the principal one accounts for two-thirds of the total material. It contains 0.043 mg of sialic acid/mg of protein, as opposed to 0.028 mg/mg for our Fetuin preparation. Its solubility in $(NH_4)_2SO_4$ and trichloroacetic acid have not been described. It is noteworthy in this connection that our Fetuin preparations are soluble in water, whereas the α-globulin of Lieberman and Ove is water-insoluble.[5]

Experiments in further fractionation of Fetuin are under way to determine the molecular distribution of the cell-stretching activity among the components of this system.

Experiments which will be reported elsewhere have demonstrated that calf fetal

serum exercises a strong stimulating action on the growth of single mammalian cells which fail to produce good colonies in conventional media.

SUMMARY

Fetuin, which exhibits a single electrophoretic boundary at pH 8.6 and is ultracentrifugally homogeneous to the extent of 95 per cent, is fully active as the factor required for attachment and stretching of S3 HeLa cells on glass. The mechanism of this action of Fetuin may be a reflection of its ability to inhibit tryptic digestion of outer-cell-wall proteins.

* Contribution No. 63. This study has been aided by a grant from the Rockefeller Foundation and from the Commonwealth Fund.

† Candidate for the Ph.D. degree in the department of biophysics. Part of this material is taken from the doctoral thesis of Mr. Fisher.

[1] G. Sato, H. W. Fisher, and T. T. Puck, *Science*, **126**, 961, 1957.

[2] H. Eagle, *J. Biol. Chem.*, **214**, 839, 1955.

[3] Data to be published.

[4] T. T. Puck, P. I. Marcus, and S. J. Cieciura, *J. Exptl. Med.*, **103**, 273, 1956.

[5] I. Lieberman and P. Ove, *Biochim. et Biophys. Acta*, **25**, 449, 1957.

[6] Thanks are due to Dr. J. T. Edsall and to Dr. D. M. Surgenor, of Harvard University, and to Drs. W. E. Ward and R. B. Houlihan, of Cutter Laboratories, for samples of highly purified albumin used in these experiments.

[7] K. O. Pedersen, *J. Phys. and Colloid Chem.*, **51**, 164, 1947; H. F. Deutsch, *J. Biol. Chem.*, **208**, 669, 1954.

[8] R. C. Parker, *Methods of Tissue Culture* (New York: Paul B. Hoeber, Inc., 1950), p. 173.

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*THE MECHANISM OF ACTION OF ALDOLASE AND THE ASYMMETRIC LABELING OF HEXOSE**

BY IRWIN A. ROSE

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Communicated by H. B. Vickery, October 15, 1957

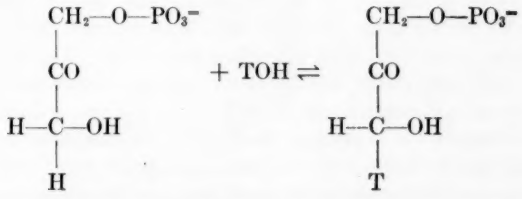
It has been suggested by Bidder[1] that the failure of methyl-labeled lactate to label carbon atoms 1 and 6 (C_1 and C_6) of glucuronic acid to an equal extent ruled out glucose as an intermediate in this interconversion, since it seemed reasonable that, in the guinea-pig liver slices they used, a symmetrically labeled glucose would have resulted from the operation of the Embden-Meyerhof scheme. Similar experiments were performed subsequently by Eisenberg,[2] in which the glucose obtained from the glycogen of liver slices was also examined, and it was reported that the glucose and glucuronic acid had similar ratios of radioactive carbon in the C_1 and C_6 positions ($C_6^{14}/C_1^{14} = 1.06$–1.2). Enzymic evidence now strongly supports the precursor role of a glucose derivative, uridine diphosphoglucose, in glucuronide synthesis.[3] However, the problem of the asymmetric labeling found is not resolved.

Schambye, Wood, and Popják[4] have reported greater labeling of C_3 compared to C_4 in the conversion of asymmetrically labeled glycerol to glucose isolated from liver glycogen of the rat. This discrepancy was greater with time following the

initial injection. In addition, Gunsalus and Gibbs,[5] in determining the distribution of isotope in the ethanol and lactic acid formed by *L. mesenteroides* acting upon C_3, and C_4-labeled glucose from the rat, obtained results which, if interpreted in terms of the accepted fermentation scheme for this micro-organism, indicate a preferential labeling of C_4 of the glucose.

Interest in this phenomenon is again stimulated by the results of Gibbs and Kandler,[6] in which a carbon-by-carbon degradation of glucose formed during short periods of photosynthesis with $C^{14}O_2$ indicated a highly asymmetric distribution of radioactivity in the glucose derived from starch or sucrose. Of particular interest was the early preferential labeling of C_4 over C_3 in experiments with *Chlorella*, tobacco leaves, and sunflower leaves. This was taken as evidence of a pathway of hexose formation other than by the condensation of two isotopically equilibrated triose phosphates.

In the review article by Korkes[7] it is suggested that experiments reported from this laboratory[8, 9] on the mechanism of action of aldolase might bear on the problem of asymmetric hexose labeling. It has been found that muscle and yeast aldolases carry out an exchange reaction between dihydroxyacetone phosphate (DHAP) and water (eq. [1]):



(1)

For the present discussion it is sufficient to conclude from this that, in the absence of G3P,[10] a compound or complex is formed between DHAP and aldolase. Stereochemical and kinetic considerations of this exchange reaction and of the over-all reactions catalyzed by the enzyme led to the conclusion that this compound, E-DHAP, may be involved directly in the further condensation reaction with G3P according to the following simplified scheme:

$$\begin{aligned} &\text{DHAP} + \text{E} \quad \rightleftharpoons \text{E-DHAP}, \\ &\text{E-DHAP} + \text{G3P} \rightleftharpoons \text{E} + \text{D-G}. \end{aligned} \tag{2}$$

Although it is not reasonable to assume that E-DHAP is the only enzyme-substrate complex formed during the complete aldolase reaction, the fact that the complex is present might permit the following exchange reaction to occur:

$$\begin{aligned} &\text{D-G} + \text{E} \quad \rightarrow \text{E-DHAP} + \text{G3P}, \\ &\text{E-DHAP} + \text{G3P}^* \rightleftharpoons \text{D-G}^* + \text{E}. \end{aligned} \tag{3}$$

Thus, in a mixture of equally labeled DHAP and G3P and unlabeled D-G, one might expect a preferential labeling of the $C_{4,5,6}$ half of the fructose diphosphate, since reaction (2) labels both halves equally, but reaction (3) labels only $C_{4,5,6}$. In order to test this hypothesis, an incubation mixture containing aldolase, DHAP, G3P, and D-G, in which the phosphate atoms of the hexose diphosphate were

equally radioactive, was analyzed to determine the rates of labeling of each triosephosphate. In addition, before isotopic equilibration had occurred, D-G was isolated and the distribution of P^{32} compared with that in the starting material.

Methods and Materials.—Muscle aldolase, DHAP, and G3P were preparations previously described.[9] P^{32}-labeled fructose-1,6-diphosphate was prepared from P^{32}-orthophosphate according to the method of Neuberg and Lustig[11] from fresh bakers' yeast (National Yeast Corp., New Haven). A 10-minute incubation was found to be sufficient for a quantitative conversion.

Analysis of the radioactivity of each phosphate radical of D-G was done in the following manner: Three tubes, each containing the equal samples of D-G, triethanolamine-HCl, pH 7.5 (25 μmoles), EDTA (1 μmole), and sodium arsenate (1 μmole) were set up in a volume of 0.9 ml. Additions were made as follows: tube 1, none; tube 2, DPN (1 μmole), triosephosphate dehydrogenase, and aldolase; and tube 3, DPNH (0.1 μmole), glycerophosphate dehydrogenase, and aldolase. Any change in optical density at 340 mμ was a measure of the amount of $C_{4,5,6}$ converted to phosphoglyceric acid (in tube 2) and $C_{1,2,3}$ converted to α-glycerophosphate (in tube 3). The assays usually agreed to within 3 per cent. The other products of these reactions are DHAP (in tube 2) and G3P (in tube 3). Both of these are hydrolyzed in alkali (1 *N* NaOH, 15 minutes, 25°) to orthophosphate[12] and could then be readily separated from the nonhydrolyzed phosphate esters by means of the Berenblum and Chain extraction procedure.[13] The increase in radioactivity in the orthophosphate fraction after enzymic treatment represents the activity of C_1-P and C_6-P, respectively, of D-G.

An alternate method for analyzing the fructose diphosphate is to treat with phenylhydrazine, which liberates C_1-P as orthophosphate.[14, 15] This was followed by the Berenblum and Chain extraction procedure, and in this way an independent determination of the radioactivity distribution was possible. The two methods were found to give results in good agreement (within 10 per cent); the phenylhydrazine method, however, requires that the D-G be isotopically pure, whereas the enzymic method, by using only the orthophosphate data, is independent of organically bound radioactive phosphate which is not hydrolyzed by the combined enzymic and alkali treatment.

In order to determine the radioactivity in DHAP and G3P present in a mixture with D-G, the treatment with enzymes and alkali is followed as above except that aldolase is omitted from the incubations. In this way, the radioactivity in DHAP is the difference in orthophosphate activity in tubes 2 and 1, and the amount of DHAP containing this radioactivity is given by the spectral change in tube 3.

The validity of these analyses depends on the purity of the enzyme preparations used. Of the enzymatic activities tested for, including aldolase, triosephosphate isomerase, triosephosphate dehydrogenase, and glycerophosphate dehydrogenase, the aldolase and triosephosphate dehydrogenase preparations were uncontaminated, and the glycerophosphate dehydrogenase was contaminated only with triosephosphate isomerase to the extent of 0.4 per cent when both activities were assayed at the concentrations of DHAP or G3P used in the experiments. All enzyme reactions were allowed to proceed for the minimum of time necessary for complete conversion of the intended substrate.

Experimental.—In order to avoid the kinetic complications brought about by

changing substrate concentrations during the experimental incubations, it is preferable to work at, or very near to, equilibrium concentrations of DHAP, G3P, and D-G. Since the equilibrium constant is a concentration term, K_{eq} = (DHAP)(G3P)/(D-G), it is possible to operate at a wide range of ratios of triosephosphate to D-G and still be at equilibrium. The value found was K_{eq} = 0.09 mM, which is in good agreement with results in the literature.[16] In order for both triosephosphates to approach the same radioactivity at isotopic equilibrium, equal concentrations of DHAP and G3P were used in all incubations. In Figures 1*a* and 1*b* are given results for incubations in which the ratio of D-G to each triosephosphate is 3.0 and 0.95. There was less than a 10 per cent change in the concentration of the components during the incubations in these experiments.

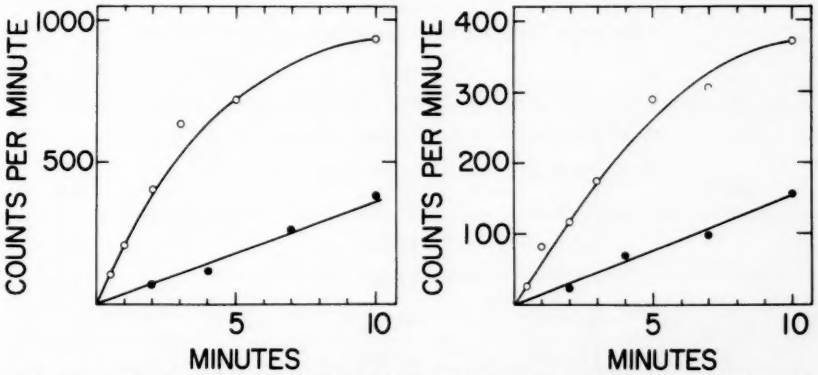


FIG. 1*a*.—Rate of appearance of radioactivity in each triosephosphate during incubation with P^{32}-labeled D-G. The incubation mixture was adjusted to pH 7.5 and contained: G3P (0.3 mM), DHAP (0.3 mM), D-G (0.9 mM, specific activity of 53,000 cpm/μmole of phosphate), triethanolamine-HCl (25 mM), EDTA (1 mM), bovine serum albumin (1 mg/ml), and muscle aldolase (0.067 units/ml). During incubation at 30°, samples of 0.2 ml. were removed, and the aldolase was inactivated by the addition of HCl. The enzymatic incubations, alkaline treatment, and orthophosphate extraction were performed on this sample as described in the text. ○ G3P, ● DHAP.

FIG. 1*b*.—Like Fig. 1*a* with the following exceptions: G3P (0.095 mM), DHAP (0.095 mM), D-G (0.10 mM, specific activity of 46,000 cpm/μmole of phosphate), and muscle aldolase (0.01 units/ml). Samples of 0.50 ml. were removed and treated as before.

A comparison of the initial rates of labeling of the triosephosphates indicates ratios of 5.3 and 4.1 in favor of G3P over DHAP. In addition to these data, D-G was isolated after 10 or 7 minutes' incubation with the enzyme and the initially unlabeled triosephosphates. The incubated samples as well as zero-time samples of D-G were analyzed for the radioactivity in each ester phosphate group. It will be noted (Table 1) that the C_6-P position lost radioactivity more rapidly than did the C_1-P position. Furthermore, a calculation of the rates of loss of radioactivity indicates ratios of ΔC_6-P to ΔC_1-P in good agreement with ratios of $G3P^{32}$ to $DHAP^{32}$ obtained from the figures for the corresponding experiment.

In work done to estimate the extent of the participation of the Embden-Meyerhof pathway and the hexose monophosphate pathway in glucose catabolism, the assumption is made that, in the former scheme, the $C_{1,\ 2,\ 3}$ and $C_{4,\ 5,\ 6}$ parts of glucose serve equally in the formation of radioactive end-products.[17] In the light of the present

data, it is more precise to restate this assumption to include the requirement that isotope equilibration exists among the compounds DHAP, G3P, and D-G during the experiment. It is clear that a failure to attain equilibration would result in a greater participation of isotope from $C_{4,5,6}$ in the formation of end-products and consequently would tend to lower the apparent participation of the hexose monophosphate pathway. The extent to which equilibrium is approached is dependent upon many factors. Perhaps one of the most important of these is the ratio G3P/D-G, since, for a high ratio, asymmetry will tend to develop in the hexose according to reaction (3). It is perhaps more likely that this ratio will be high under conditions of hexose synthesis from exogenous substrates than under conditions of glucose breakdown.

TABLE 1

EFFECT OF ALDOLASE ON DISTRIBUTION OF ISOTOPE IN D-G

Exp.	Sample Counted	Specific Activity (cpm/μatom)	Specific Activity with Time (cpm/μatom)	$\frac{\Delta C_6\text{-P}}{\Delta C_1\text{-P}}$
Fig. 1*a*	C_1-P, 0′	52,800	1,300	5.5
	C_1-P, 10′	51,500		
	C_6-P, 0′	57,000	7,200	
	C_6-P, 10′	49,800		
Fig. 1*b*	C_1-P, 0′	44,000	3,700	4.1
	C_1-P, 7′	40,300		
	C_6-P, 0′	47,800	15,200	
	C_6-P, 7′	32,600		

It is not possible at present to state whether the results obtained with the isolated aldolase system can be applied as an explanation of any or all cases of asymmetric labeling of glucose. The data are suggestive, however, in that, over the range of concentration ratios tested, the asymmetry observed with aldolase alone is greater than that observed in vivo.

Summary.—Isotope experiments with crystalline muscle aldolase have demonstrated an exchange reaction between $C_{4,5,6}$ of fructose-1,6-diphosphate and glyceraldehyde-3-phosphate. These results are offered as a possible explanation for the asymmetric labeling of glucose in biological systems.

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*ACTIVATION OF HYDROGEN TRANSFER BETWEEN PYRIDINE NUCLEOTIDES BY STEROID HORMONES**

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It will be shown in this paper that a soluble enzyme from human placenta promotes the transfer of hydrogen between two pyridine nucleotide coenzymes and that this reaction is activated by minute quantities of certain steroid hormones. This enzyme system catalyzes the over-all reaction:

$$\text{TPNH} + \text{DPN}^+ \rightarrow \text{TPN}^+ + \text{DPNH},$$

where DPN and TPN refer to the oxidized forms of di- and triphosphopyridine nucleotides and DPNH and TPNH designate the respective reduced nucleotides. Consideration will be given to the pivotal role of this reaction in the control of metabolic processes. The implications of the control of pyridine nucleotide balance by steroids will be discussed in relation to the physiological actions of these hormones.

Recent experiments of Villee *et al.*[1, 2, 3] showed that, in the presence of isocitrate the reduction of DPN catalyzed by crude extracts of placenta was accelerated by certain steroids. These findings were interpreted in terms of a steroid activation of a DPN-specific isocitric dehydrogenase. The observations of Villee were readily confirmed in this laboratory. However, it was found that the response of crude placental extracts to steroids was variable and that the remarkable stimulation of the reduction of DPN by certain hormones disappeared upon fractionation of the extracts. We have observed that the addition of *catalytic* quantities of TPN to the reaction mixture increased markedly the ability of different placental preparations to respond to steroids and restored this effect in preparations which were inactive in this respect. It will be shown that the apparent stimulation of a DPN-linked isocitric dehydrogenase can be accounted for in terms of a coupling of the

TPN-specific isocitric dehydrogenase of placenta with the action of a soluble transhydrogenating system here described:

$$\text{Isocitrate} + \text{TPN}^+ \rightleftarrows \alpha\text{-ketoglutarate} + \text{CO}_2 + \text{TPNH} + \text{H}^+ \qquad (a)$$

$$\text{TPNH} + \text{DPN}^+ \rightleftarrows \text{TPN}^+ + \text{DPNH} \qquad (b)$$

$$\text{Isocitrate} + \text{DPN}^+ \rightleftarrows \alpha\text{-ketoglutarate} + \text{CO}_2 + \text{DPNH} + \text{H}^+$$

Only step *b*, the transhydrogenase system, is activated by steroids. Isocitric dehydrogenase is not the only TPN-specific enzyme which can act as a generator for TPNH in this coupled reaction. It can, for instance, be replaced equally well by glucose-6-phosphate dehydrogenase.

Partially purified fractions from placenta which catalyze hydrogen transfer between the two forms of pyridine nucleotide also catalyze the oxidation of steroids by DPN and TPN. Of the steroids examined, only those which can undergo oxidoreduction by pyridine nucleotide-linked hydroxysteroid dehydrogenases[4] are capable of activating the transhydrogenase mechanism. The experiments are consistent with the view that the proteins catalyzing dehydrogenation and transhydrogenation are identical.

METHODS

Preparation of Placental Enzymes.—Crude extracts were prepared by the method of Villee and Gordon.[2, 3] Human placenta was obtained immediately after birth and placed on ice. As much blood as possible was removed by perfusion with approximately 1 liter of isotonic sodium chloride. The tissue was homogenized with 4–5 volumes of ice-cold 0.25 *M* sucrose, either in a glass homogenizer equipped with a Teflon pestle or in a Waring Blendor. All further operations were carried out at less than 2°. The homogenates were centrifuged at 2,000 × *g* for 15 minutes, and the resulting supernatant fluid was centrifuged at 59,000 × *g* for 1 hour in a Spinco preparative ultracentrifuge. The supernatant fluid obtained from the second centrifugation was stored either in an ice-bath or in the frozen state and will be referred to as the "crude placental extract." Fractionation was carried out with ammonium sulfate at 0°. The precipitates which formed upon the addition of various amounts of ammonium sulfate were collected by centrifugation and were dissolved in 0.01 *M* tris(hydroxymethyl)aminoethane (Tris) buffer of pH 7.4. These fractions were stored at −10°.

Determinations.—Spectrophotometric measurements were made with a Beckman model DU spectrophotometer using either Pyrex or silica cells of 1 cm. light path. The formation of reduced pyridine nucleotides was followed by measurement of change in absorbance at 340 mμ. The molar extinction coefficient of reduced pyridine nucleotides at 340 mμ was assumed to be 6,220. The TPN was estimated with purified isocitric dehydrogenase of rat heart in Tris buffer at pH 9.0 in the presence of an excess of isocitrate and 0.0003 *M* $MnCl_2$. The isocitrate was determined in the same system with an excess of TPN. Glucose-6-phosphate and DPN were estimated with the aid of yeast glucose-6-phosphate and alcohol dehydrogenases, respectively. Protein was determined by the method of Warburg and Christian.[5]

Materials.—DPN and TPN were commercial products of greater than 85 per cent purity and were uncontaminated by each other. Isocitric dehydrogenase,

purified from rat heart by a slight modification of the method of Siebert *et al.*,[6] was generously donated by S. K. Wolfson, Jr. This purified enzyme did not reduce DPN upon the addition of isocitrate and $MnCl_2$ in the presence or absence of estradiol 17β. It had a specific activity of 0.6 μmole of isocitrate oxidized per minute per milligram protein under the conditions of the experiments to be described. Twice recrystallized yeast alcohol dehydrogenase was obtained as a paste suspended in 60 per cent saturated ammonium sulfate solution from the Sigma Chemical Company, St. Louis, Missouri. It was diluted with ice-cold deionized water just prior to use. The samples of estradiol 17β and of testosterone were of greater than 98 per cent purity as determined by enzymatic assay.[7] The other steroids and diethylstilbestrol were of high purity. All other preparations were of commercial origin.

RESULTS

Response of Crude Extracts of Placenta to Estradiol 17β.—The experiments were carried out under conditions slightly different from those described by Gordon and Villee.[2, 3] Each vessel contained 300 μmoles Tris buffer of pH 7.4, 1 μmole of DPN, and 1.0 ml. of a crude extract of placenta equivalent to approximately 0.2 gm. of fresh tissue. The total volume was made up to 3.0 ml. with water, and estradiol 17β was added in 0.01 ml. of dioxane, which was also added to the control vessels.

The reduction of DPN by some crude placental extracts was accelerated markedly by estradiol 17β at a final concentration of 5×10^{-6} M, with or without the addition of isocitrate (1×10^{-3} M) or glucose-6-phosphate (1×10^{-3} M) to the reaction mixture. Under similar conditions, other placental preparations did not respond to estradiol 17β, but it was possible to restore their sensitivity to this hormone by the addition of *catalytic* amounts (0.01–0.05 μmole) of TPN.

Further experiments with crude placental extracts revealed that (*a*) these preparations reduced TPN very rapidly in the absence or presence of added substrates (the reduction of TPN was unaffected by estradiol 17β); (*b*) the magnitude of the increase in rate of DPN reduction induced by estradiol 17β was enhanced by the addition of TPN-reducing substrates, such as isocitrate or glucose-6-phosphate; and (*c*) even with those extracts of placenta in which the steroids accelerated the reduction of DPN in the absence of exogenous TPN, the addition of catalytic amounts of the latter nucleotide magnified the stimulation induced by estradiol 17β.

These phenomena are explicable in terms of a coupled reaction between a TPN-specific isocitric dehydrogenase or a TPN-specific glucose-6-phosphate dehydrogenase and an enzymatic mechanism which could transfer hydrogen from TPNH to DPN (see eqs. [*a*] and [*b*]). Presumably those extracts which were stimulated by estradiol 17β without the further addition of catalytic amounts of TPN contained sufficient quantities of the latter nucleotide to allow the coupled reaction to proceed. The amount of TPN required was found to be very small, since as little as 0.01 μmole of TPN restored the ability of inactive placental extracts to respond to estradiol 17β. Definitive evidence in support of this hypothesis required enzyme preparations free from endogenous substrates and TPN. Extracts of acetone powders of the placental enzymes were not suitable, but fractionation with ammonium sulfate provided a simple method for removing interfering substances.

Experiments with Fractionated Enzymes.—The material precipitated from crude placental extracts by the addition of ammonium sulfate to 40 per cent saturation contained active, TPN-linked isocitric and glucose-6-phosphate dehydrogenases, and a steroid-sensitive pyridine nucleotide transhydrogenating system. It was devoid of endogenous substrates and pyridine nucleotides. The experiment depicted in Figure 1 shows that, over a 45-minute period without added substrate,

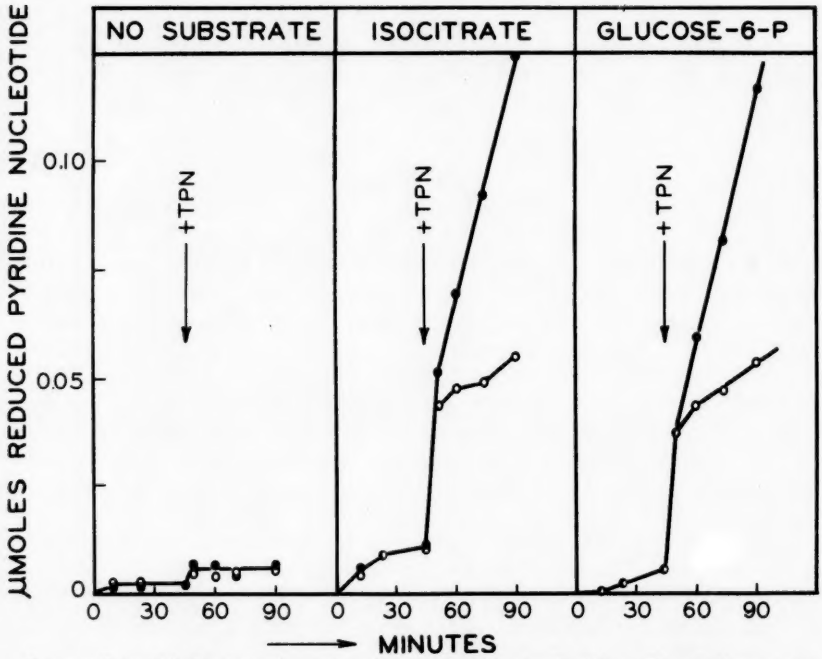

FIG. 1.—Estradiol-stimulated formation of reduced pyridine nucleotides. The reaction systems contained in 3.0 ml. the following ingredients: 300 μmoles Tris pH 7.4; 1 μmole DPN^+; 1 μmole $MnCl_2$; and 4.2 mg. protein of a 30–40 per cent saturated ammonium sulfate fraction of placenta. Paired cuvettes were run with (●●●) or without (○○○) 4 μg. estradiol 17β in 0.01 ml. dioxane. Parallel experiments were carried out without added substrate (*left*), with 1.5 μmoles isocitrate (*center*), and with 3 μmoles glucose-6-phosphate (*right*). At time 45 minutes, 0.04 μmole TPN was added to all cuvettes. Optical measurements at 340 mμ in cuvettes of 1 cm. light path. Temp. 25°.

this preparation did not reduce DPN, whether estradiol 17β (5×10^{-6} *M*) was present or not. Furthermore, the addition of 0.04 μmole of TPN after 45 minutes of incubation did not induce a reduction of DPN during the subsequent 45 minutes. When isocitrate was included in the incubation mixture, DPN was not reduced with or without the further inclusion of estradiol 17β. Later addition of TPN led to an instantaneous and complete reduction of the nucleotide by the highly active TPN-linked isocitric dehydrogenase in the enzyme preparation. In the absence of hormone, the subsequent change in absorbance at 340 mμ was very small over the following 45 minutes. Howevet, with both isocitrate and extradiol 17β present, DPNH gradually accumulated after all the TPN had been converted to TPNH.

This experiment is in full accord with a stimulation by estradiol 17β of a mechanism which transfers hydrogen from TPNH to DPN. A corollary of this hypothesis would be that any other substrate which could reduce TPN in this system should substitute for isocitrate. This proved to be the case with glucose-6-phosphate. Figure 1 shows that DPN was not reduced by glucose-6-phosphate unless catalytic amounts of TPN were added, in which case the further reduction of DPN was stimulated by estradiol 17β.

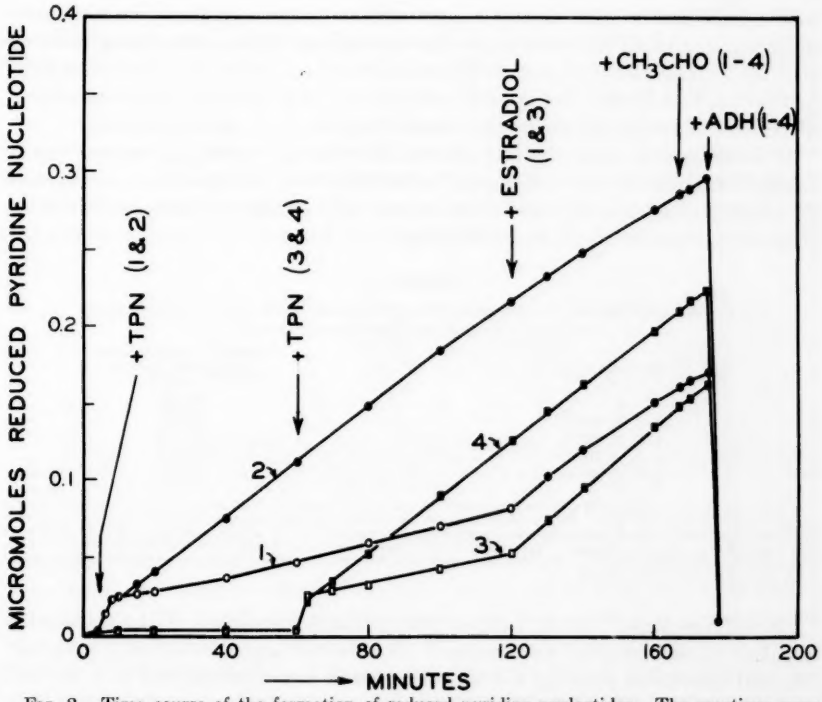

FIG. 2.—Time course of the formation of reduced pyridine nucleotides. The reactions were carried out in 3.0 ml. systems containing 300 μmoles Tris pH 7.4; 1 μmole DPN; 1 μmole $MnCl_2$; 1.5 μmole sodium isocitrate; 107 μg. purified rate heart isocitric dehydrogenase; and 1.92 mg. protein of a 0–35 per cent saturated ammonium sulfate fraction of a placental extract. Cuvettes *2* and *4* contained 4 μg. estradiol 17β in 0.01 ml. dioxane initially. TPN (0.023 μmole) was added at 5 minutes to cuvettes *1* and *2*. 4 μg. estradiol 17β was added to cuvettes *1* and *3* at 120 minutes. All cuvettes received 10 μmoles acetaldehyde in 0.01 ml. at 167 minutes and an excess of yeast alcohol dehydrogenase at 175 minutes. Optical measurements at 340 mμ against a control containing enzyme and buffer. Temp. 25°.

These considerations are amplified further by the findings summarized in Figure 2. The activity of the TPN-specific isocitric dehydrogenase in the enzyme preparation used in this experiment was rather feeble, so an excess of this TPN-reducing enzyme was added. All the cells contained isocitrate, DPN, and purified isocitric dehydrogenase. The DPN was not reduced under any circumstances unless TPN (0.02 μmole) was also present. If the TPN was added 5 minutes after initiation of the reaction, it was reduced instantaneously. Without estradiol 17β, very little

reduced pyridine nucleotide accumulated above the expected amount of TPNH, whereas a rapid formation of reduced nucleotide took place in cells containing the hormone. Similar changes were manifest if the TPN was added 60 minutes after the beginning of the reaction. The delayed addition of estradiol 17β also increased greatly the accumulation of reduced pyridine nucleotide in cells to which TPN had been added after 5 or 60 minutes of incubation. Finally, it will be seen that when acetaldehyde was added at the end of the experimental period, the rate of change in absorbance in cells containing TPN and estradiol 17β was unimpeded. But the further addition of yeast alcohol dehydrogenase caused a virtually instantaneous disappearance of all the reduced pyridine nucleotide. This is convincing evidence that the increase in absorbance at 340 mμ which took place after all the added TPN had been reduced reflected a gradual accumulation of DPNH and not of some other ultraviolet-absorbing product (e.g., reduced nicotinamide mononucleotide).

It is noteworthy that the steroid-sensitive transhydrogenating system can be readily fractionated with ammonium sulfate and that it is relatively stable, since its activity remains unimpaired after storage at 2° for several days and is not destroyed by repeated freezing and thawing.

TABLE 1*

EFFECT OF VARYING CONCENTRATIONS OF ESTRADIOL 17β ON STIMULATION OF PYRIDINE NUCLEOTIDE TRANSHYDROGENATION

Estradiol 17β (M)	Total Reduced Pyridine Nucleotide Formed (μmoles)
0	0.034
1.2×10^{-8}	0.042
1.2×10^{-7}	0.114
1.2×10^{-6}	0.158
4.9×10^{-6}	0.134

* Each cuvette contained 300 μmoles Tris buffer of pH 7.4; 1 μmole $MnCl_2$; 1.5 μmole sodium isocitrate; 1 μmole DPN; 0.023 μmole TPN; 107 μg. of purified rat heart isocitric dehydrogenase and ammonium sulfate fraction from placenta (0–35 per cent saturation) containing 1.92 mg. protein, in a final volume of 3.01 ml. Estradiol 17β was added in 0.01 ml. dioxane. The reaction was initiated by the addition of the placental fraction. Time 100 minutes. Temperature 25°. The rate of formation of reduced pyridine nucleotide was approximately linear with time over the period studied.

Steroid Specificity.—Table 1 shows that maximal stimulation of the transhydrogenating system occurred with estradiol 17β at a final concentration of 1×10^{-6} M. Marked activity of estradiol 17β was observed at a concentration of 1×10^{-7} M, and a small but detectable effect of this hormone could be demonstrated at 1×10^{-8} M.

The reactivity of various ammonium sulfate fractions toward other steroids differed somewhat from one preparation to another. At steroid concentrations of about 5×10^{-6} M, the transhydrogenating mechanism was stimulated by estradiol 17β and estrone, but not by estradiol 17α or by diethylstilbestrol. At a steroid concentration of 3×10^{-5} M, stimulation of the reaction was observed with testosterone, but not by 17α-methyltestosterone or by cortisone. The transhydrogenating mechanism stimulated by testosterone appeared to be more labile than that which was activated by estradiol 17β.

Reduction of Pyridine Nucleotides by Steroids.—Fractions of placenta obtained by precipitation between 0 and 40 per cent saturation of ammonium sulfate reduced DPN in the presence of *substrate amounts* of estradiol 17β, in confirmation of the studies of Langer and Engel.[8] The relatively crude enzyme was found to react

almost equally well with DPN and TPN. Table 2 shows that, under conditions similar to those used to study the transhydrogenating system and at a steroid concentration of 2.5×10^{-5} *M*, 10–20 mμmoles of estradiol 17β were oxidized per hour per milligram of protein. Although, by necessity, these measurements of the hydroxysteroid dehydrogenase activity were carried out at higher steroid concentrations than were used in the transhydrogenase experiments, it has been observed that the rates of the estradiol 17β-activated transhydrogenation and of the reduction of pyridine nucleotides by estradiol 17β are of the same order of magnitude. In a specific instance, 1 mg. of protein of an ammonium sulfate fraction of placenta at pH 7.4 catalyzed the oxidation of estradiol 17β (2.5×10^{-5} *M*) at a rate of 59 mμmoles per hour with DPN (3.3×10^{-4} *M*). The transhydrogenase activity of the same preparation at pH 7.4 (3.3×10^{-4} *M* DPN; 1.3×10^{-5} *M* TPN; and 4.9×10^{-6} *M* estradiol 17β), with isocitric dehydrogenase as the reducing system, was 10.9 mμmoles per hour per milligram protein. The differences in steroid concentrations involved in this comparison are probably of no great influence on the reaction rates, since at least the bacterial 17β-hydroxysteroid dehydrogenase has a Michaelis constant for estradiol 17β far less than 1×10^{-6} *M*.[9]

TABLE 2*

OXIDATION OF ESTRADIOL 17β BY AMMONIUM SULFATE OF PLACENTAL FRACTIONS

Ammonium Sulfate Fraction (Per Cent Saturation)	Millimicromoles Reduced Pyridine Nucleotide Formed per Hour per Mg. Protein		Relative Rates
	DPN	TPN	DPN/TPN
0–30	15.2	8.2	1.85
30–40	21.5	12.5	1.72

* The activities were measured by observing the reduction of pyridine nucleotide at 340 mμ in systems of 3.0 ml. volume containing 300 μmoles Tris pH 7.4; 1 μmole DPN or TPN; 0.074 μmole estradiol 17β in 0.01 ml. dioxane, and appropriate amounts of enzyme. Readings were made at intervals against control cuvettes containing all ingredients except estradiol 17β. Temperature 25°.

Inhibition by Mercuric Ions.—Low concentrations of mercuric ions inhibited the enzymatic reduction of TPN and DPN by estradiol 17β. The degree of inhibition of the transhydrogenation reaction by Hg^{++} was found to be of the same order of magnitude. The latter reaction was carried out with a sufficient excess of added isocitric dehydrogenase. Whereas 2×10^{-5} *M* $HgCl_2$ had little effect on both reactions, brief preincubation of the enzyme with 4×10^{-5} *M* $HgCl_2$ inhibited both the hydroxysteroid dehydrogenase and the transhydrogenating reactions by 80–90 per cent. The placental and certain other hydroxysteroid dehydrogenases are known to be inactivated by heavy-metal ions and have been presumed to be sulfhydryl group containing proteins.[4, 8, 10]

DISCUSSION

It is evident from these experiments that the increased rate of reduction of DPN promoted by steroid hormones in crude extracts of placenta is explicable in terms of an activation of the transfer of hydrogen from TPNH to DPN by steroids. Thus it is not necessary, as others have,[2, 3] to postulate the separate existence of a DPN-specific isocitric dehydrogenase as responsible for these hormonal effects.

There is considerable evidence that DPN and TPN serve different metabolic functions and that the natural occurrence of these two different pyridine nucleotides is of profound functional significance. TPNH can act as a reducing agent in a

number of synthetic reactions which take place outside the mitochondria and in which DPNH cannot participate. Examples of such biosynthetic pathways are (*a*) the synthesis of fatty acids, where TPNH acts as a specific reductant in fatty acyl dehydrogenase reactions,[11, 12] and (*b*) the entry of one-carbon fragments into serine[13] and into purines[14] catalyzed by a series of folic acid-dependent enzyme systems which utilize TPNH as a specific hydrogen donor.[15]

It is indeed remarkable that studies on the metabolic concomitants of the action of steroid hormones upon accessory sexual tissues[16] have shown that these same extra-mitochondrial synthetic reactions, which specifically require TPNH, are extremely sensitive to the action of steroids. Thus Mueller[16] found that, shortly after the administration of estradiol 17β to the ovariectomized rat, the incorporation of one-carbon fragments (derived from a variety of precursors) into serine and into the purines of nucleic acids in the uterus was increased immensely. In similar experiments, estradiol 17β stimulated the conversion of acetate to fatty acids and to cholesterol but did not influence the oxidation of acetate.

Furthermore, a number of biochemical changes in the accessory organs of reproduction of the male induced by testosterone could have, as their common denominator, a change in the balance between TPNH and DPNH. Androgenic steroids initiate and support the accumulation and secretion of fructose and citric acid in some male accessory sexual tissues.[17] The synthesis of fructose by these organs involves the reduction of glucose to sorbitol by TPNH, followed by the DPN-linked oxidation of sorbitol to fructose.[18, 19] The over-all conversion of glucose to fructose thus simulates the action of pyridine nucleotide transhydrogenase insofar as there is a stoichiometric transfer of hydrogen from TPNH to DPN in this process. Those lobes of the prostate gland which accumulate and secrete citrate in response to steroids do, in fact, possess the enzymatic machinery for the oxidation of citrate by the tricarboxylic acid cycle, involving the action of a TPN-specific isocitric dehydrogenase.[20] Citrate—and no other organic acid—probably accumulates in response to steroids because the unfavorable TPNH/TPN ratio acts to brake the isocitric dehydrogenase reaction and prevents the rate of oxidation of citrate from keeping pace with its rate of synthesis.[21] Again, the synthesis of fatty acids from acetate in the prostate gland is most sensitive to testosterone.[22]

In all mammalian tissues examined, including accessory sexual organs, TPNH is present in far higher concentrations than TPN, whereas the steady-state level of DPN is usually greater than that of DPNH.[23] Hence the ratio

$$\frac{[\text{TPNH}]\,[\text{DPN}]}{[\text{TPN}]\,[\text{DPNH}]}$$

is always very high. This quotient is the equilibrium constant of the pyridine nucleotide transhydrogenase reaction. The vast majority of pyridine nucleotide-linked dehydrogenases employ only one type of hydrogen acceptor; they do not function equally well with TPN and DPN. Any disturbance in the balance between TPNH and DPNH by a transhydrogenating mechanism would alter the nice equilibrium between synthesis and degradation required for the orderly growth and function of cells. Since there is not a uniform intracellular distribution of pyridine nucleotides and of enzymes with which they react, the factors which determine the steady-state levels of the reduced and oxidized forms of these coenzymes will

vary in different regions of the cell.[11] Similar considerations concerning the regulation of the balance between hydrogenation and phosphorylation have been advanced by Hoch and Lipmann.[24]

The existence of enzyme systems which catalyze the transfer of hydrogen between the two natural forms of pyridine nucleotide is well documented. Kaplan *et al.*[25] have purified a soluble transhydrogenase, apparently a discrete protein, from *Pseudomonas fluorescens.* In mammalian species, the transfer of hydrogen between the oxidized and reduced forms of TPN and DPN, between certain unnatural pyridine nucleotides, and even from DPNH to DPN has been observed heretofore only with mitochondrial preparations from some, but not all, tissues.[26, 27] The mitochondrial transhydrogenase system has not been purified extensively. It is by no means clear whether the hydrogen transfer from one pyridine nucleotide to another carried out by mitochondria is catalyzed by a single enzyme or reflects the operation of a coupled reaction, whereby an enzyme with dual nucleotide specificity alternately oxidizes and reduces an intermediate, present in minute amounts and thereby permits a rapid net transfer of hydrogen from one pyridine nucleotide to another.

Kaplan[28] has suggested that the mitochondrial pyridine nucleotide transhydrogenase system plays an important role in regulating the balance between various synthetic and energy-yielding reactions. His experiments suggest that, while the oxidation of DPNH by liver mitochondria serves as a source of energy for the synthesis of adenosine triphosphate, the oxidation of TPNH is not coupled with phosphorylation unless hydrogen is first donated from TPNH to DPN by the mitochondrial transhydrogenase reaction.

In our studies, those steroid hormones which accelerate the transfer of hydrogen between two forms of pyridine nucleotide also reduce both DPN and TPN in the presence of the same soluble placental enzymes. The enzyme activities are associated in certain protein fractions and absent from others. Both activities are inhibited by similar concentrations of Hg^{++} to approximately the same extent. Furthermore, the activities are of comparable magnitude in the preparations studied. The simplest interpretation of these experimental findings is that the reversible oxidation and reduction of the steroids themselves constitute the steroid-activated transhydrogenating mechanism. Consider, for example, the case of estradiol 17β. The rapid conversion of this steroid to estrone with either TPN or DPN as a hydrogen acceptor would establish an equilibrium mixture of estrone and estradiol 17β. We visualize the transfer of hydrogen from TPNH to DPN as taking place according to the following equations:

$$\begin{array}{l} H^+ + TPNH + \text{estrone} \rightleftarrows TPN^+ + \text{estradiol } 17\beta \\ \text{Estradiol } 17\beta + DPN \rightleftarrows \text{estrone} + DPNH + H^+ \\ \hline TPNH + DPN^+ \rightleftarrows TPN^+ + DPNH \end{array}$$

This reaction is made possible by the dual nucleotide specificity of the dehydrogenating enzyme.

The placental hydroxysteroid dehydrogenase which oxidizes estradiol 17β is not alone in this class of enzymes in having dual nucleotide specificity. A soluble 3α-hydroxysteroid dehydrogenase isolated from liver reacts with both DPN and

TPN.[10] Recent experiments[29] have shown that liver microsomes of various species contain a firmly bound 3α-hydroxysteroid dehydrogenase. These particles also interconvert hydrocortisone and cortisone by the action of a specific 11β-hydroxysteroid dehydrogenase. Both microsomal enzymes react at comparable rates with DPN and with TPN.

Detailed studies of the specificities and substrate affinities of highly purified, adaptive hydroxysteroid dehydrogenases of bacteria have revealed that these enzymes bind certain steroids tenaciously.[9,30] The magnitudes of the Michaelis constants correspond with the concentrations at which many steroids exert their physiological actions. Moreover, the binding of steroids by these enzymes is very sensitive to even minor structural alterations of the steroid molecule. A number of striking similarities have been shown to exist between the molecular features necessary for physiological activity and those which favor efficient binding of the steroid to the enzyme surfaces.[9, 30] The observation that these enzymes exhibit both the high affinity and the specificity for steroids demanded by hormonal activity led to the suggestion that hydroxysteroid dehydrogenases are intimately concerned with the hormonal action of steroids.[9, 30] The importance of the state of oxidation of steroids had already been pointed out by Huggins and his co-workers[31] from considerations of the relationship of molecular structure to the growth-promoting activity of these hormones.

The present experiments strongly suggest that steroids can participate in transhydrogenation between pyridine nucleotides. On the assumption that the enzyme in placenta which catalyzes the reduction of TPN and DPN by estradiol 17β is the same as that which promotes the steroid-activated transfer of hydrogen from TPNH to DPN, it is possible to regard the pyridine nucleotides as substrates for the transhydrogenation, and the steroid as a coenzyme for the latter reaction. In this way it becomes possible to assign a functional role to the mammalian hydroxysteroid dehydrogenases which possess dual nucleotide specificity. In the presence of truly minute amounts of steroids, they act as mediators of hydrogen transfer between pyridine nucleotides.

It would appear that the thermodynamic properties of steroid hormones are well suited for the promotion of hydrogen transfer in such systems. Equilibrium constants for the interconversion of hydroxy- and ketosteroids have recently become available from measurements made with highly purified bacterial hydroxysteroid dehydrogenases.[30] The free-energy changes involved in these reactions are such as to favor the existence of significant amounts of both oxidized and reduced forms of steroid at physiological hydrogen ion concentrations. Thus, at pH 7 and at 298° K., the equilibrium ratio of 4-androstene-3,17-dione to testosterone is in the vicinity of 0.4, assuming that the oxidized and reduced pyridine nucleotides are present in equimolar amounts. The calculated change in free energy for the interconversion of these two steroids is thus $\Delta F^\circ = 5.2$ Kcal/mole. For several 3-hydroxysteroids, the equilibrium ratio of ketone to alcohol varies from 0.01 to 0.07 ($\Delta F^\circ = 6.0$–7.2 Kcal/mole) under the same conditions. The oxidation of a steroid hydroxyl group to a ketone with a pyridine nucleotide as a hydrogen acceptor involves the stoichiometric liberation of hydrogen ion; hence the equilibrium is strictly dependent upon pH. It follows that the ability of steroids to effect transhydrogenation by their reversible oxido-reduction would vary in a predictable manner with the hydrogen

ion concentration. For example, at pH 9.0 and in the presence of the appropriate hydroxysteroid dehydrogenase and equimolar concentration of oxidized and reduced pyridine nucleotide, 17β hydroxysteroids would exist in the ketone form to an extent of 97 per cent. Thus, at pH 9.0, one would not expect the steroid-hydroxysteroid dehydrogenase system to function as an efficient mediator of transhydrogenation. Similar considerations would hold if the hydrogen ion concentration was too far removed from neutrality on the acid side. If the present interpretation of our findings is correct, these deductions are in agreement with the finding of Gordon and Villee[2] that the activation of the reduction of DPN in crude extracts of placenta by estradiol 17β is maximal at pH 7.3.

It may be emphasized that the oxidation of the 17β-hydroxyl group of steroids gives, at neutrality, an equilibrium ratio of alcohol to ketone closer to 1 than that of any other steroid oxido-reduction which has been studied.[30] It is, perhaps, not altogether fortuitous that the 17β-hydroxyl function is of paramount importance for the biological activity of estrogenic and androgenic hormones.

The few known mammalian pyridine nucleotide-linked enzymes which exhibit dual nucleotide specificity with non-steroidal substrates, e.g., glutamic dehydrogenase[32] and glucose dehydrogenase,[33] may not be expected to function as efficiently as hydroxysteroid dehydrogenases to effect a transfer of hydrogen from TPNH to DPN, or vice versa, at pH 7. The equilibria which they catalyze are unfavorable, and the affinities for their substrates are too low in this respect.

All types of *naturally occurring* steroid hormones undergo a variety of oxido-reductions in animal tissues, and the enzymes catalyzing these transformations are widely distributed in nature.[4] A number of these enzymes possess the properties requisite for them to act as transhydrogenating systems between pyridine nucleotides and, possibly, between other coenzymes engaged in hydrogen transport. From the limited evidence at hand, it appears that these enzymes differ considerably in their affinities and specificities for steroids and in their intracellular and tissue distribution. Thus some of the enzymes which permit the oxidation or reduction of oxygen substituents on steroids may have significance not only as mechanisms for the inactivation of hormones[4] but also as catalysts for the interconversion of the oxidized and reduced forms of hydrogen-transporting coenzymes. For this reason, further studies on the properties and distribution of the family of enzymes which catalyze the oxido-reduction of steroids in animal tissues are of the utmost importance. The fact that certain steroid hormones exert a profound action in more than one physiological domain may well relate to the lack of rigid specificities of various hydroxysteroid dehydrogenases.

SUMMARY

Soluble enzyme preparations of human placenta promote the transfer of hydrogen from TPNH to DPN in the presence of minute amounts of certain steroid hormones. The same enzyme preparations catalyze the oxidation of these steroids by both forms of pyridine nucleotide. Evidence is presented for the identity of a placental hydroxysteroid dehydrogenase with the transhydrogenase activity.

It is proposed that the metabolic function of hydroxysteroid dehydrogenases with dual pyridine nucleotide specificity is to act as pyridine nucleotide transhydrogenases. Properties of some hydroxysteroid dehydrogenases which favor transhydro-

genation are: (1) their high affinity and specificity for particular steroids; (2) their ability to react with both DPN and TPN; and (3) the suitable equilibria between steroid alcohols and ketones which obtain at physiological hydrogen ion concentrations.

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† Scholar in Cancer Research of the American Cancer Society.

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*CONDUCTANCE OF TETRABUTYLAMMONIUM TETRAPHENYLBORIDE**

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A correlation between the Arrhenius-Ostwald classification of electrolytes into strong and weak and the curvature of their phoreograms with respect to the Onsager tangent was immediately noticed when the slope of the tangent was first calculated.[1] Most salts and mineral acids and bases in aqueous solution give phoreograms which approach the limiting tangent from above (anabatic[2]), while most organic acids and bases in water and nearly all salts in nonaqueous solvents of dielectric constant substantially lower than that of water give curves which approach the tangent from below (catabatic) as concentration decreases toward zero. The generally accepted explanation of the strongly catabatic curves was based on ion association.[3] Until recently, however, it has not been possible to analyze theoretically data, the phoreograms of which were relatively near the Onsager tangent; if anabatic, the only applicable hypothesis was that of zero ion pair concentration, while, if mildly catabatic, it was realized that the calculated association constants could have little physical significance because the method of calculation forced the association constant to absorb higher terms from the electrophoresis and relaxation effects in mobility.

Two significant changes have occurred recently in the theoretical situation; first, the necessity of including ion association (in principle at least, even if unobservable within the present limits of precision of measurement) as an inevitable consequence of a model in which ions are assigned a nonzero size was recognized,[4] and, second, the problem of the explicit statement of the higher terms in mobility has been solved for the limiting case of low ($\kappa a < 0.2$) but nonzero concentrations.[5] It is therefore possible now to analyze conductance data in the formerly inaccessible region where the curves went from anabatic to catabatic as the dielectric constant was decreased. The present theory predicts that anabatic curves can be observed in solvents of dielectric constant appreciably less than that of water, provided that the ion size is large enough; in other words, curves similar to those of the alkali halides in water should be observable in nonaqueous systems. The purpose of this paper is to present preliminary data for several examples of such systems.

The current model used to represent ions is the charged sphere-in-continuum. It is, of course, only a first approximation, but, until its limitations have been established, it seems pointless to consider more complicated models. Quaternary ammonium ions with large substituents furnish cations which have essentially spherical symmetry and in which the charged central atom is electrically well insulated. Anions of similar structure have long been sought; the spherical anions, such as halide ions, are relatively small compared to most solvent molecules, while the physically larger anions usually have an exposed and rather concentrated charged site or else are not spherical. The tetraphenylboride ion, which has recently become available as a quantitative reagent for potassium,[6] offers an anion

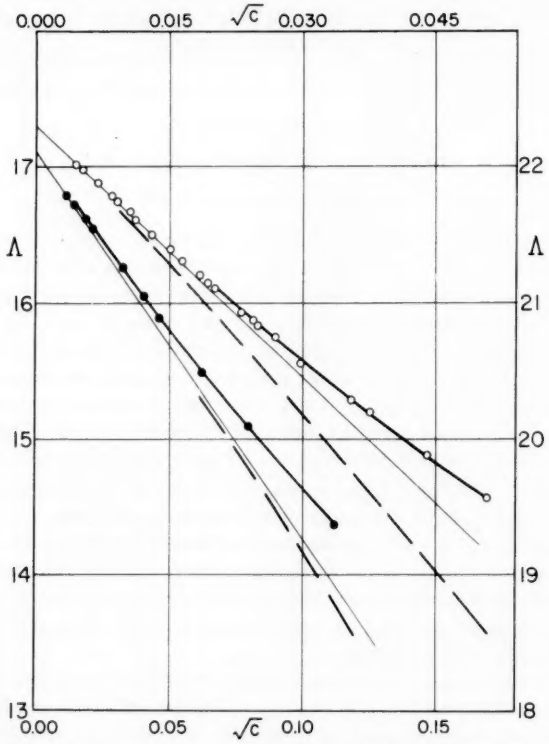

FIG. 1.—Phoreograms: propylene carbonate, *solid circles*, co-ordinates left and below; nitrobenzene, *open circles*, co-ordinates right and above.

which is practically an ideal partner for the tetrabutylammonium ion. The quaternary tetraphenylborides are easily prepared by precipitation, on mixing dilute aqueous solutions of sodium tetraphenylboride and of quaternary halide, and can be readily recrystallized from acetone-water mixtures. The high molecular weight (561.72) permits accurate preparation of dilute solutions.

For low concentrations of salts in which association is slight, the conductance equation assumes the approximate limiting form

$$\Lambda = \Lambda_0 - S\sqrt{c} + Ec \log c + J_1 c - K_A \Lambda_0 c - F\Lambda_0 c, \qquad (1)$$

where S is the Onsager coefficient, E and J_1 are the Fuoss-Onsager coefficients, K_A is the association constant (formerly[4] designated by A) and $Fc = 5\phi/2$ with ϕ equal to the volume fraction of solute. The coefficient E is independent of ion size, and, since $c < 1$ in the relevant concentration range, the theoretical reference curve,

$$\Lambda_r = \Lambda_0 - S\sqrt{c} + Ec \log c,$$

always lies *below* the Onsager tangent. The linear term, $J_1 c$, is positive and, except at extremely low concentrations, is numerically larger than the transcendental

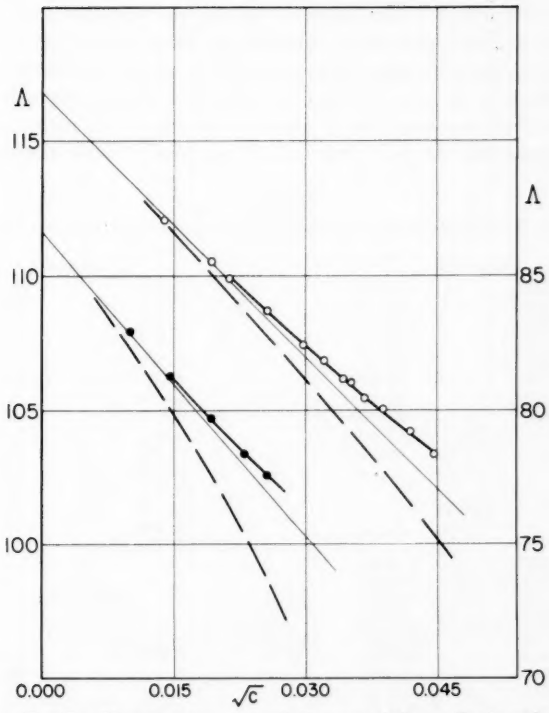

FIG. 2.—Phoreograms: acetonitrile, *open circles*, ordinate scale left; acetonitrile-carbon tetrachloride mixture, *solid circles*, ordinate scale right.

term; hence, if ion association is slight, the conductance curve will lie above the limiting tangent. Now, combining the suggestions of Denison and Ramsey[7] and of Gilkerson,[8] we have

$$K_A = K_0 e^b; \qquad b = e^2/aDkT. \tag{2}$$

Consequently, as the dielectric constant, D, decreases, the originally anabatic phoreogram drops and crosses the tangent, becoming catabatic over its entire course in the range of dilute solutions, due to ion association. But if b is small enough, $K_A\Lambda_0 c$ will not be greater than $(J_1 c + Ec \log c)$; if D is decreased, b can still be kept small by increasing a. Tetrabutylammonium tetraphenylboride in solvents with dielectric constants down to at least 20 corresponds to such a case, as shown in Figures 1 and 2. The experimental data are summarized in Table 1.

In the figures, the light solid line is the Onsager tangent, while the dashed line gives Λ_r. As the dielectric constant decreases in the sequence propylene carbonate (65.1), acetonitrile (36.0), nitrobenzene (34.82), and acetonitrile–carbon tetrachloride (55.3 wt. per cent CCl_4, $D = 21.9$), it will be seen that the reference curve, Λ_r, drops more and more rapidly away from the limiting tangent. Nevertheless, the observed curves lie above the limiting tangent, and therefore $J_1 c > |Ec \log c -$

$K_A\Lambda_0 c|$ for these systems. The curves in nitrobenzene and in acetonitrile closely resemble the curves for potassium chloride in water; in fact, if Λ/Λ_0 is plotted against κa for all three systems, these normalized curves are nearly indistinguishable. Consequently, we conclude that the KCl-H_2O pattern of conductance is not a special type characteristic of only aqueous solutions, but rather is merely one member of a broad class of electrolytic solutions, namely, those in which b is small.

TABLE 1

CONDUCTANCE OF TETRABUTYLAMMONIUM TETRAPHENYLBORIDE IN VARIOUS SOLVENTS AT 25°

$10^4 c$	Λ	$10^4 c$	Λ
$MeC_2H_3CO_2$		$C_6H_5NO_2$	
1.319	16.79	1.261	21.61
2.033	16.71_5	1.723	21.50
3.564	16.60_5	2.265	21.39
4.691	16.53_5	2.708	21.31
10.426	16.26	3.774	21.14
16.326	16.05	4.087	21.10
21.493	15.88	5.416	20.93
38.60	15.49_5	5.997	20.87
64.11	15.09	6.293	20.84
125.84	14.37	7.285	20.75
		8.962	20.58
MeCN		12.664	20.29
		14.138	20.20
		19.454	19.88
		25.735	19.56_5
1.966	112.0_5		
3.734	110.5_5		
4.559	109.9	MeCN-CCl_4*	
6.610	108.7		
6.769	108.6		
8.924	107.4	0.938	82.9
10.357	106.8	2.138	81.2_5
11.741	106.2	3.708	79.7
12.398	106.0	5.338	78.3_5
13.576	105.4	6.563	77.5
15.111	105.0		
17.629	104.2		
19.844	103.3_5		

* 55.3 wt. per cent CCl_4.

The strong dependence of the coefficients E and J_1 on dielectric constant and viscosity is shown in Figure 3, where $\delta\Lambda = (\Lambda - \Lambda_r)$ is plotted against concentration. As the dielectric constant decreases, both E and J_1 increase almost as D^{-3}; since the ordinate scale is in Λ-units, the curves also become steeper as viscosity decreases. (In the sequence of the lines in the figure from top to bottom, the viscosities are 0.00423, 0.00330, 0.01834, and 0.02553.) The fact that the plots are linear shows that the $Ec \log c$ term adequately represents the transcendental terms[5] in the relaxation field over the given range of concentration and that the $c^{3/2}$ terms are negligible within the present (rather large) experimental error.

If the assumption is made that ion association is negligible for the above systems, then the slopes of the $\delta\Lambda$-c plots evaluate $(J_1 - F\Lambda_0)$. In order to obtain the hydrodynamic coefficient, F, viscosities of solutions of the salt were measured in several solvents (acetonitrile, acetonitrile–carbon tetrachloride mixture containing 51.0 wt. per cent of the latter, and isobutyronitrile). The viscosity increment over that of the solvent is made up of two terms at low concentrations, an electrostatic term, $\eta_0\delta\eta_1$, and a term due to the volume occupied by the solute. We assume that the

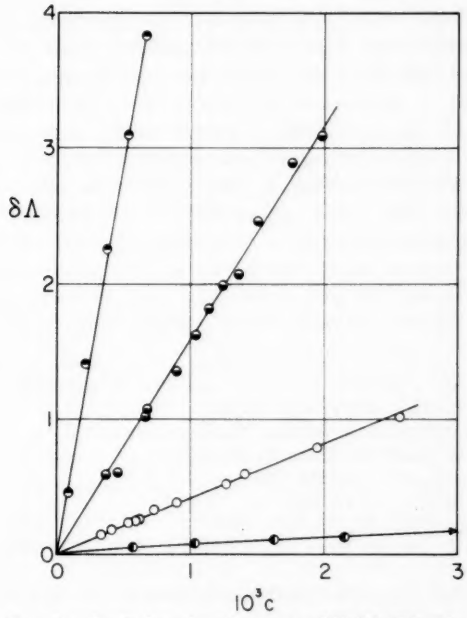

FIG. 3.—Deviation function: ◓, CCl_4-MeCN mixture; ◒, MeCN; ○, $C_6H_5NO_2$; ◐, $MeC_2H_3CO_3$.

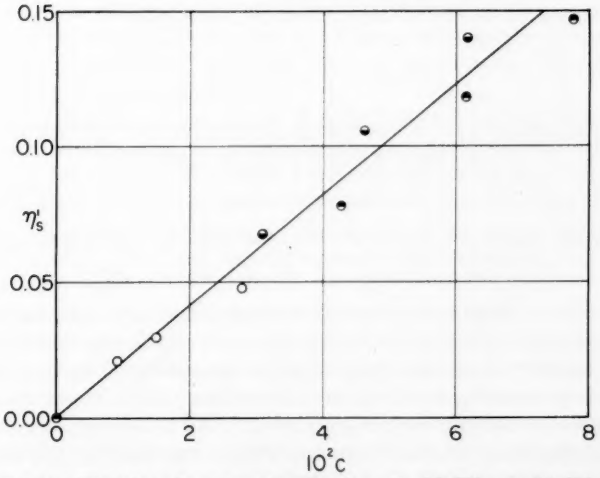

FIG. 4.—Einstein volume term in viscosity: ◒, MeCN; ◓, MeCN-CCl_4; ○, *i*-BuCN.

latter has the value $5\phi/2$ calculated by Einstein, where ϕ is the volume fraction of solute. In Figure 4 is shown a plot of η_s', where

$$\eta_s' = (\eta/\eta_0) - 1 - \delta\eta_1 = 5\phi/2 = Fc,$$

where η is solution viscosity and η_0 is solvent viscosity; $\delta\eta_1$ was calculated by the Falkenhagen formula.[9] The slope of the line which averages the points gives $F = 2.0$. If we assume that anion and cation are equal in size, with radius R, then $(8\pi R^{3/3})\ (Nc/1{,}000) = Fc$, whence $R = 5.40 \times 10^{-8}$ cm. This value agrees well with the dimensions estimated from molecular models of the ions. Finally, the electrostatic radius can be evaluated, now that F is known. For propylene carbonate, $\mathring{a} = 5.5$, which is excellent agreement. For the other solvents, however, the following values were found: nitrobenzene, $\mathring{a} = 7.6$; acetonitrile, $\mathring{a} = 6.2$; acetonitrile–carbon tetrachloride ($D = 21.9$), $\mathring{a} = 7.5$. This dependence of apparent ion size on solvent may mean that there is some interaction between solute and solvent in the process of ion pair formation. Further work, now in progress, in solvents of lower dielectric constant will be needed in order to decide this question.

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† Du Pont Postdoctoral Research Fellow, 1956–57.

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MODIFICATION OF CELL-MIGRATION PATTERNS IN THE EARLY CHICK EMBRYO

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In recent years there have been several indications of a relationship between acetylcholine (Ach) synthesis and pacemaker activity in the vertebrate and invertebrate heart[1–4] which have made clear the desirability of studying the development of Ach-synthesizing mechanisms in embryonic heart. One way of attacking this problem is to assay for choline acetylase activity in the early developing heart, to determine whether an increase in such activity is correlated in time with the onset of spontaneous contractions. Such a study is currently in progress in this laboratory. A second point of attack is represented by the possibility of influencing chemically the synthesis, or at least the activity, of choline acetylase in the developing tissue. Since there is evidence from studies on adult mammalian heart that the activity of this enzyme may be enhanced by its reaction products[5] (i.e., is an autocatalyzed enzyme), it did not seem unreasonable to expect to see some effect

on hearts developing in the presence of exogenous Ach. Therefore, a series of experiments was undertaken in which chick embryos were explanted before any sign of cardiac development and were cultured in vitro during the entire period of heart formation, in the presence of Ach.

In the course of this phase of the investigation, it was noted that, in a fairly high percentage of the cases, anomalous development occurred, consisting for the most part of the development of double hearts. The purpose of the present report is to describe and discuss these defective embryos.

Materials and Methods.—White Leghorn chicks were explanted from the egg at Hamburger-Hamilton stages 4–5 (primitive streak to head process).[6] The technique of New, recently described in full detail,[7] was utilized. In this method the blastoderm and a large circle of its associated vitelline membrane are removed from the yolk, inverted, and cultured in a watch glass on a pool of egg-albumen. Thus the normal close relationship between the blastoderm and the vitelline membrane is retained. Since the chick grows with its hypoblast, or ventral side, uppermost, the formation of the heart and endodermal structures can be visualized clearly.

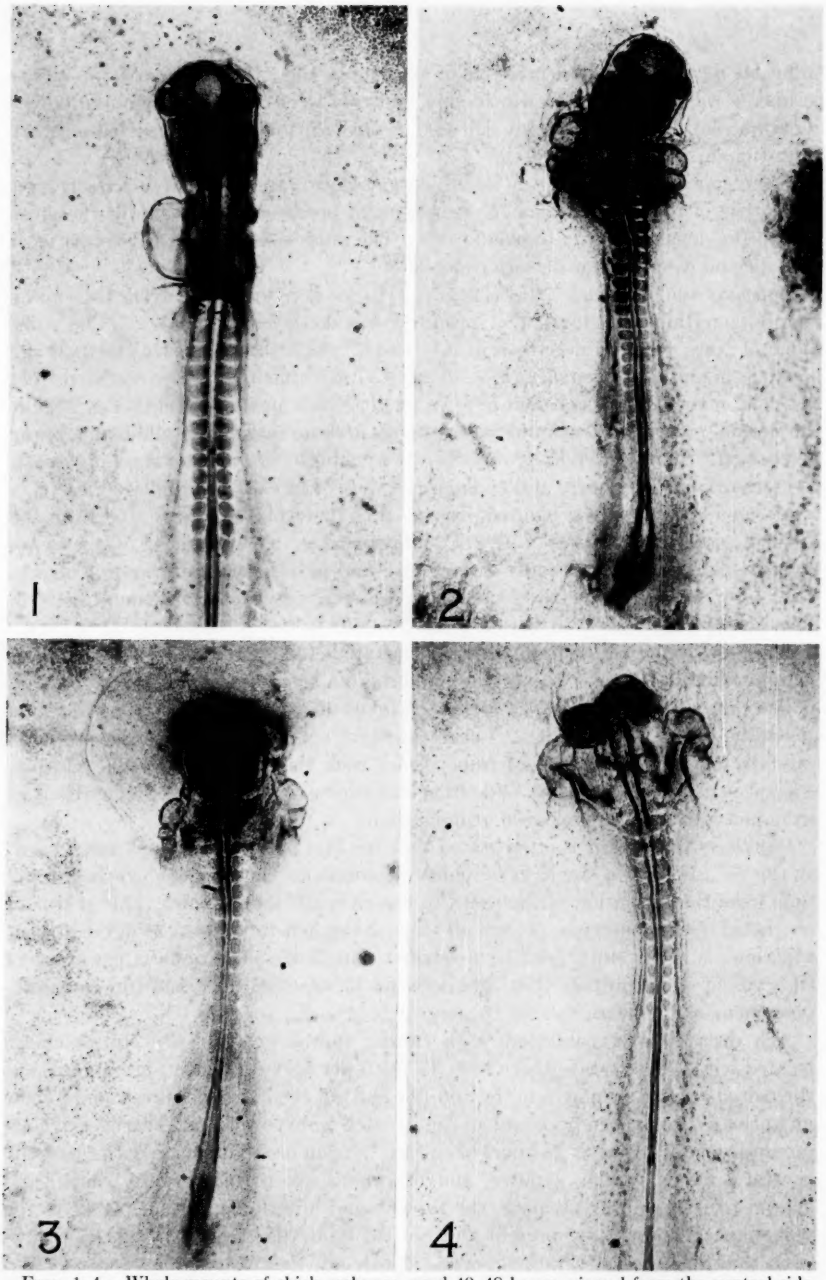
After an embryo was explanted, excess saline and yolk were aspirated from the surface, and a small crystal of Ach or a control substance was placed on the hypoblast in the area opaca, usually in line with and anterior to the primitive streak. Thus no mechanical effect could be exerted directly on the embryonic area itself. The Ach crystals dissolved within a few seconds in the surrounding tissue fluids, flooding the entire blastoderm with concentrated Ach. Control crystals included sodium chloride, sucrose, quartz sand, and sodium acetate.

The cultures were incubated a further 24 hours at 38° C., by which time they had generally reached stages 10–12 (10–16 pairs of somites). They were examined carefully under a dissecting microscope and were then fixed in Bouin's solution, stained in alcoholic cochineal, and either mounted as whole-mount preparations or sectioned for further histological examination.

Results.—Although it is often stated that the first beats of the chick heart occur at the $9^1/_2$–10-somite stage, in actuality spontaneous contractions can begin any time from the formation of the ninth to the eleventh somite pair. This is true of untreated control embryos. It was also true of the Ach-treated chicks in the present experiments. Therefore, pending a detailed statistical analysis of a larger group of embryos, it would appear that Ach does not hasten the process of functional development of the heart.

Ach does, however, interfere with cardiac morphogenesis. Out of 43 chicks treated with Ach crystals, there were 17 (39.5 per cent) in which normal fusion of the paired cardiac primordia in the mid-line had not occurred. Figures 1 and 2 show an untreated control embryo and an Ach-treated embryo with two hearts, as whole-mount preparations after 24 hours of culture. It can be seen that, in both cases, the central nervous system, somites, and notochord are normal in size, shape, and spatial relations. Furthermore, the foregut and anterior intestinal portal in the experimental embryo are present and not too badly misshapen. It is clear, however, that the anterior intestinal portal is located farther rostrad than normally and that the hearts have formed at either side of it.

Figures 3 and 4 show embryos with progressively more severe effects of the Ach. Figure 3, showing a slightly younger chick than those shown above, exhibits an



FIGS. 1–4.—Whole mounts of chick embryos, aged 40–48 hours, viewed from the ventral side. ×17. Fig. 1, Untreated control embryo. Fig. 2, Cardia bifida embryo produced by treatment with a crystal of acetylcholine; mild response. Note the mirror symmetry between right and left hearts. Fig. 3, Cardia bifida embryo produced as in Fig. 2. More marked response. Note the anterior intestinal portal arching under the mid-brain. Fig. 4, Cardia bifida embryo produced as in Fig. 2. Severe response, involving abnormalities of the foregut and brain.

animal with a normal nervous system, notochord, and somites. However, the foregut is represented only by a short, wide, crescentic fold under the midbrain. It should be noted that the hearts themselves are located at about the normal anteroposterior position, extending from the level of the second somite to the anterior part of the hindbrain.

The animal shown in Figure 4 demonstrates a still greater effect of the Ach, in which organs other than the heart and foregut are involved. In this interesting "cardia bifida" embryo, the spinal cord, somites, and even the otic cup appear to be in excellent condition; however, the foregut is almost nonexistent, and the entire brain is markedly stunted. In this animal, as in those above, the hearts are only slightly farther rostrad than would be the case if they had fused in the mid-line.

An examination of the sectioned material confirms the relations seen in the whole-mounts. Figure 5 shows four sections, spaced at approximately 0.1-mm. intervals, through the head end of an embryo similar to the one seen in Figure 4. Figure 5, *a*,

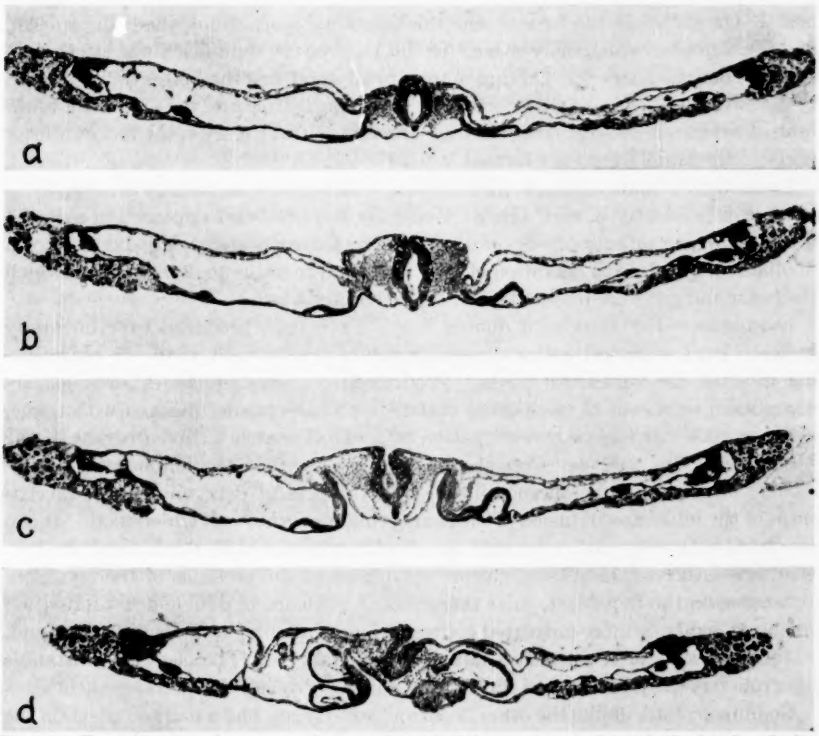


FIG. 5.—Ten-micron sections, spaced approximately 0.1 mm. apart, through the head end of a cardia bifida embryo of the degree of severity of that in Fig. 4. ×35. *a*, Section through the first somite. *b*, Section through the posterior "foregut" region. *c*, Section through the hindbrain. *d*, Section through the stunted midbrain.

is a section through the first somite. This animal is slightly younger than those shown in the whole-mounts, having only eleven pairs of somites. In a control em-

bryo at a similar stage, a section at this level would pass just posterior to the anterior intestinal portal. The well-formed dorsal aortae and omphalomesenteric veins can be identified, and the neural tube, except for its somewhat vesicular appearance, seems normal. Figure 5, *b*, through what should be the posterior foregut region of the specimen, shows the first signs of an endodermal in-folding to form the sides and roof of the primitive pharynx. Note the thickening of the splanchnic mesoderm over the omphalomesenteric veins.

One-tenth of a millimeter farther anterior (Fig. 5, *c*), a section through the unclosed groove of the hindbrain shows the more definitive invagination of the endoderm to form the roof and sides of the "foregut." The right omphalomesenteric vein has now given way to a clearly recognizable double-walled heart. The dorsal aortae are flattened dorsoventrally; however, when these structures are traced farther rostrad, no sign of aortic arches is to be seen.

The final section (Fig. 5, *d*), which in a control embryo would cut through the rostral portion of the ventricle or the bulbus cordis, shows the definitive double heart. On each side can be seen the thickened epimyocardium, the endocardium, and the reticular cardiac-jelly space. In this embryo the right heart was larger than the left, but the latter was beating more strongly and was the better differentiated of the two, as evidenced by the formation of a looped structure, the upper and lower limb of which can be seen in the section. As was seen in Figure 4, the more anterior parts of the brain are poorly formed.

In the cardia bifida chicks, before fixation, both hearts were usually beating, in all cases, independently of each other. Generally the left heart appeared to have the faster, stronger pulsation. Several of the treated embryos which did not form double hearts suffered a condition of what appeared to be omphalocephaly, in which the heart and gut were found anterior to the stunted brain.

Discussion.—Embryos with double hearts have been produced experimentally by many workers, investigating various aspects of development, in species representing most of the vertebrate phyla. Production of these anomalies has been accomplished by means of mechanical obstruction of the cardiac anlagen with tissue, agar, or glass barriers or by extirpation of a mid-line area. (For a review of the literature in this field, see Copenhaver.[8]) More recently the influence of direct-current electric fields has been utilized.[9] In the present experiments, the mechanism of the inhibition of fusion of the heart primordia is not well understood. It can be stated with some assurance that the effect is not caused by mechanical obstruction, due either to the general culture conditions or the presence of the crystal of substance on the hypoblast, since there was no evidence of double heart formation in any of eighty or more untreated control explants, nor did crystals of quartz sand, sodium chloride, or sucrose have any effect on embryos. The last two substances also rule out the possibility of an osmotic imbalance being the effective agent.

Sodium acetate, unlike the other "control" substances, had a marked effect on the chicks, similar to that of Ach. Out of twenty chicks treated with sodium acetate crystals, seven (35 per cent) developed into cardia bifida embryos, more or less well formed, except for their double hearts. Acetate, however, is much more generally deleterious than is Ach, since nine of the acetate-treated embryos showed no development at all or were completely disaggregated. This total disaggregation of some of the embryos by acetate suggested the possibility that a calcium-binding

phenomenon might be involved. It has long been known that acetate ion forms weak organometallic compounds with heavy metals.[10] Similarly, there is evidence in the literature that Ach may exert some of its physiological and pharmacological effects by liberating calcium from cell surfaces.[11] It is thus conceivable that these two agents, Ach and acetate ion, by making calcium ion (to some extent) unavailable to the endodermal and mesodermal cells of the embryo, disturb the normal intercellular relationships sufficiently to prevent migration and fusion of the cardiac anlagen.

It must be emphasized that this hypothesis is tentative. However, preliminary experiments with known calcium-binding agents such as citrate and ethylenediamine tetra-acetic acid seem at present to be confirmatory.

Several investigators have shown that normal cardiogenesis is dependent upon, or at least related to, the presence of endoderm or its derivatives.[8, 12, 13, 14] In contrast, Rudnick has reported the formation of pulsatile cardiac masses from chick epiblast, in the complete absence of hypoblast.[15] At present, it is difficult to state whether the cardiac effects reported here resulted from a direct action of the Ach on the heart-forming areas or were only secondary to a disruption of the normal patterns of endodermal development. Experiments in progress are designed to determine which of the germ layers is primarily affected, to elucidate the mechanism of the effect, and to identify and quantify the causal agents. The ability to do controlled studies of intercellular relations and morphogenetic movements by chemical or pharmacological intervention in normal development is a highly desirable goal from the point of view of the experimental embryologist.

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*CHROMOSOMAL POLYMORPHISM IN CARIBBEAN ISLAND POPULATIONS OF DROSOPHILA WILLISTONI**

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In *Drosophila willistoni*, the amount of chromosomal polymorphism measured as the mean number of heterozygous inversions per fly in a population was found by da Cunha, Burla, and Dobzhansky,[1] and verified by others,[2, 3] to be generally proportional to the diversity of ecological niches available to the particular population. Recently Dobzhansky[4] examined samples of this species from Central America and the Caribbean islands and concluded that (*a*) the amount of chromosomal polymorphism measured either as the mean number of heterozygous inversions per fly or as the number of different kinds of inversions in an island was generally proportional to the size of the island and thus to the diversity of ecological niches probably available to the population and (*b*) this insect species most likely populated the Greater Antilles from South and Central America by chance dispersion over the same routes as those Darlington[5] and Simpson[6] hypothesized to have been used by the land animals of these islands.

As Dobzhansky[4] pointed out, most of the flies examined in his recent study were several generations removed from the wild females from which they were descended. All those representing island populations were cultured 7–10 months in the laboratory. Then the different strains from within an island were intercrossed strains A × B, B × C, C × D, D × E, etc., to produce the single larva cytologically determined from each cross. Samples from two or more localities within each of four of the eight islands represented were pooled. For two of the four islands of the Greater Antilles, the determinations were made on such composite samples; yet only one of these, that for Jamaica, exceeded a total of 36. Although circumstances necessitated these methods of treatment, the procedures were different from those of the earlier studies[1–3], wherein the cytological determinations were made on one larval offspring of each female inseminated in the wild, and samples from different localities were not pooled. It seems greatly desirable to determine whether or not Dobzhansky's recent results are really comparable with the earlier ones, as well as to extend his observations where possible, and to note in any island the presence of any inversion not already reported therefrom.

Materials and Methods.—I obtained and analyzed a fairly large sample (100) from the Agricultural Experiment Station, Rio Piedras, Puerto Rico, and a small sample (21) from El Yunque National Park, Puerto Rico, in June, 1953; another small sample (19) from the William Beebe estate, Trinidad, in January, 1955; and other very small samples (less than 12 each) from Ciudad Trujillo, Dominican Republic, and Port-au-Prince, Haiti, in September, 1952, and from Constant Spring, Jamaica, in August, 1952. All these Caribbean samples were given the older, conventional treatment described in the earlier papers.[1–3]

Mean Frequency of Heterozygous Inversions.—The mean frequencies of the heterozygous inversions per fly, with their standard errors, found in my Caribbean samples, except in those from Haiti and Jamaica, are given in the left-hand columns of

Table 1. For purposes of comparison, the values obtained by Dobzhansky[4] in these islands are given in the right-hand columns of the same table.

The two samples from Trinidad reported in the left- and right-hand columns of Table 1 were collected at about the same time of the year in 1955, in January and February, respectively. Dobzhansky's sample was made up almost entirely of flies from Arima Valley.[7] My sample was collected in the mountains on the Beebe estate. The mean frequency of heterozygous inversions per fly is significantly greater in Dobzhansky's sample.

The two different samples reported from Hispaniola in Table 1 were for flies collected at different times of the year, nearly 3 years apart, and in rather distant parts of the island (about 260 km. apart) at markedly different elevations (a difference of more than 400 meters). In spite of these differences, the mean frequency of heterozygous inversions per fly did not differ significantly.

TABLE 1

MEAN NUMBER OF HETEROZYGOUS INVERSIONS PER FLY

Locality	Sex	Frequency	Locality	Sex	Frequency
Ciudad Trujillo, Dom. Republic	♀	2.25 ± 0.32	Haiti*	♀	1.84 ± 0.22
	♂	1.67 ± 0.27		♂	2.45 ± 0.39
Rio Piedras, Puerto Rico	♀	1.24 ± 0.12	Puerto Rico*	♀	1.28 ± 0.21
	♂	1.34 ± 0.13		♂	1.09 ± 0.29
El Yunque, Puerto Rico	♀	1.60 ± 0.21			
	♂	1.17 ± 0.31			
Beebe Estate, Trinidad	♀	1.50 ± 0.15	Trinidad*	♀	3.18 ± 0.29
	♂	1.14 ± 0.40		♂	2.58 ± 0.29

* Th. Dobzhansky, *Evolution*, **11**, 280–293, 1957.

Data summarized separately for Rio Piedras (collecting site was near sea level) and El Yunque (collecting site was at an elevation of about 900 meters), Puerto Rico, in the left-hand columns of Table 1 show that the mean number of heterozygous inversions per fly did not differ significantly from one locality to the other (distance about 28 km.). Although Dobzhansky's[4] Puerto Rican sample and mine were collected at different times of the year, February and June, respectively, nearly 3 years apart, it appears most reasonable to judge the reliability of the newer procedures by comparing these samples rather than those from Hispaniola or those from Trinidad, because Dobzhansky's Puerto Rican sample included only flies from Rio Piedras and El Yunque (10 flies from the former and 26 from the latter[7]) and had therefore the same origins as mine. Also the largest sample from any of these three islands was from Puerto Rico. Comparison of the Rio Piedras and El Yunque means in the left-hand columns of Table 1 with Dobzhansky's mean from Puerto Rico, in the right-hand columns of Table 1, shows that they did not differ significantly.

These comparisons suggest that in an island with a relative impoverishment of gene arrangements (12 different inversions known in Hispaniola and 10 in Puerto Rico) the mean frequency of heterozygous inversions per fly differs very little, if at all, from locality to locality, elevation to elevation, or season to season. Such relative uniformity may result in part from the close association that *D. willistoni* often has with man in these heavily populated islands; *willistoni* sibling species are found abundantly in many yards and gardens throughout the Greater Antilles[8]. Although this relative uniformity needs to be verified with more and larger samples,

it appears that the newer procedures used by Dobzhansky[4] are about as suitable as the older ones when applied to samples from the Greater Antilles. On the other hand, regional samples from Trinidad, with its relative abundance of different gene arrangements (19 now known), must be combined with considerable caution, if at all, because the mean number of heterozygous inversions per fly likely differs in different localities in this island. Similar caution should be observed in the treatment of samples from other islands in very close proximity to the South American continent and its highly variable populations.

Distribution of Different Kinds of Inversions.—Table 2 lists the frequencies (in per cent) of the various heterozygous inversions in my samples from Rio Piedras, El Yunque, Beebe estate, and Ciudad Trujillo. The frequencies of inversions not

TABLE 2

FREQUENCIES (IN PER CENT) OF HETEROZYGOUS INVERSIONS IN FOUR SAMPLES FROM THE ANTILLES

Inversion	Origin			
	Ciudad Trujillo	Rio Piedras	El Yunque	Beebe Estate
XL Chromosome				
None	75.0	96.6	100.0	100.0
A	...	*1.7*	...	...
D	*25.0*	*1.7*	...	...
XR Chromosome				
None	100.0	100.0	100.0	100.0
Females studied	8	59	15	12
IIL Chromosome				
None	63.6	56.0	33.3	73.7
A or B	27.3	43.0	57.1	4.8
D or E	18.2	8.0	14.2	*19.0*
F	9.1	...	...	...
IIR Chromosome				
None	36.4	57.0	61.9	68.4
A	27.3	*18.0*	*9.5*	...
E	54.5	30.0	28.6	31.6
III Chromosome				
None	45.5	73.0	61.9	26.3
A or B	...	*1.0*	...	...
C	9.1	...	...	...
J	45.4	26.0	38.1	47.4
L	...	...	...	15.8
M	...	...	...	5.3
L + M	...	...	...	5.3
Flies studied	11	100	21	19

found in Dobzhansky's[4] samples from the respective islands are indicated in italics. The most notable of these latter inversions included in this table is XL-D. Dobzhansky[4] found it in all his Central American samples, but he did not find it in samples from any of the Antilles except Trinidad. It has previously been reported from Cuba.[2] Now it is known from all the Greater Antilles, except Jamaica, the island from which Dobzhansky's largest sample was obtained. The size of his samples from the Lesser Antilles, while not precluding the presence of this inversion in those islands, does lessen the probability of its occurrence there. Thus this inversion appears like others, including IIIA-1 and III-F, to have invaded the Greater Antilles from the northwestern mainland of South America or Central America and therefore to fit the Darlington-Simpson hypothesis.

In addition to the inversions listed in Table 2, it should be noted that my very

small sample from Port-au-Prince, Haiti, included one fly with the compound IIR-D + E. The IIR-D has previously been reported only to occur rarely in several localities in the Amazon basin[1, 3] and in a single fly each from Mogi, São Paulo,[1] and from Lake Placid, Florida.[2] Da Cunha and Dobzhansky[3] have pointed out that such a widely disjunct distribution suggests the possibility that two or more cytologically similar inversions are involved. It might, however, be remarked that there also exists the possibility that a single inversion has recurred and become established more than once in the history of the species. At any rate, the inversion reported as IIR-D in Haiti and Florida is very likely the same.

The distributions of the four other inversions reported from the various islands for the first time in Table 2 do not particularly support Dobzhansky's extension of the Darlington-Simpson hypothesis, but neither do they contradict it.

The total number of different kinds of heterozygous inversions now reported from each of the Greater Antilles and Florida is Cuba 13, Hispaniola 12, Jamaica 10, Puerto Rico 10, and Florida 11. While the rank orders of size of island and of number of kinds of inversions present correspond completely, it is not clear that the differences in number of kinds of inversions are real. This is because of the very few populations sampled in each island and of the generally small sizes of the samples. Even if the differences are real, the presence of a greater number of kinds of inversions in a somewhat larger oceanic island than in a somewhat smaller one may very well reflect the greater probability of chance immigrants landing in quantities sufficient for establishment in the larger island rather than the greater diversity of ecological niches available there. The number of different kinds of inversions in island populations is clearly less than that generally found in continental populations of Central and South America. However, for the present at least, it appears to be more fruitful to compare mean numbers of heterozygous inversions in one island with those in another.

Summary.—Quantitative results obtained by pooling regional samples collected within an island and kept in small mass cultures for several months in the laboratory are found to be comparable with results from unpooled samples of F_1 larvae from wild females, if such pooling is restricted to samples from the Greater Antilles. The data obtained from both procedures suggest that the mean frequency of heterozygous inversions per fly in *D. willistoni* may differ very little from locality to locality, elevation to elevation, or season to season within an island of the Greater Antilles and that this relative uniformity is associated with a comparative impoverishment of different kinds of gene arrangements and perhaps also with a close association of the species with man. The distribution of another inversion (XL-D) is described as supporting Dobzhansky's[4] extension of the Darlington-Simpson hypothesis of certain migration routes for population of the Antilles.

* Collecting in the Greater Antilles was supported in part by a grant from the Penrose Fund of the American Philosophical Society. Transportation to and from Trinidad was provided by the Campanha National de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) of Brazil, and collecting in Trinidad was supported in part by a grant from the Rockefeller Foundation. It is a pleasure to acknowledge the hospitality and many courtesies extended me by Dr. W. G. Downs and other members of the staff of the Trinidad Regional Virus Laboratory.

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ON THE CONCEPT OF SURFACE INTEGRAL

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By surface $S = (T, A)$: $p = p(w)$, $w \in A$, $p = (x, y, z)$, $w = (u, v)$, is meant a continuous mapping from an admissible set A of the uv-plane E_2 into the p-space E_3 (or E_N)[1]; by area of S is meant the Lebesgue area; by τ_r, $r = 1, 2, 3$, are meant the orthogonal projections of E_3 into the oriented co-ordinate planes yz, zx, xy; by (T_r, A), $q = q_r(w)$, $w \in A$, is meant the mapping $T_r = \tau_r T$, $r = 1, 2, 3$.

Suppose (T, A) has finite area. Then for every $G \subset A$ open in A, we denote by $V(T, G)$, $V(T_r, G)$, $V^+(T_r, G)$, $V^-(T_r, G)$ the area of (T, G), the total variation (or area) of (T_r, G), the total positive and negative variations of (T_r, G), respectively, as defined by Cesari[1] by consistent use of the topological indices of the continuous closed curves which are the images under T, T_r of cycles traced on A.

Let $f(p, t)$, $p = (x, y, z)$, $t = (t_1, t_2, t_3)$, be any function defined on $T(A) \times R$, where R is the unit sphere $t_1^2 + t_2^2 + t_3^2 = 1$. Suppose, in addition, that f is bounded and uniformly continuous on $T(A) \times R$. Then, on the base of the same topological considerations mentioned above, Cesari has defined[1, 2] the surface integral

$$J(T, A, f) = \int_S f(p, t)$$

as the limit of Weierstrass-like sums.

The existence of J as a limit, the invariance of J with respect to Fréchet equivalence, and the lower semicontinuity of J under general conditions of regularity are proved by Cesari.[1, 2] The integral J has been used in the direct method of calculus of variations by Cesari, and J. Cecconi, L. M. Danskin, A. Sigalov. General formulations of the Gauss-Green and Stokes theorems have been given by J. Cecconi, using J.

If the generalized Jacobian vector, $J(w) = (J_1, J_2, J_3)$, $w \in A$, is defined in A as in surface-area theory, then the Lebesgue-Tonelli integral,

$$I(T, A, f) = \int_A f[p(w), J(w)]\, dw,$$

exists, and the equality,

$$J(T, A, f) = I(T, A, f),$$

holds[2] for every (T, A) of finite area, provided that T_r, $r = 1, 2, 3$, are all absolutely continuous mappings (AC mappings).

We shall now define another integral $H(T, A, f)$ whose coincidence with J holds for all mappings (T, A) of finite area (i.e., even not AC). For any $p \in T(A)$

consider the components g of $T^{-1}(p)$ in A. Let $\mathfrak{M}_0$ be the class of all subsets of A which are unions of such components g. Let $\mathfrak{G}_0$ and $\mathfrak{B}_0$ be the class of all sets in $\mathfrak{M}_0$ which are open in A, and Borel measurable, respectively. For any set $K \in \mathfrak{M}_0$ let $\varphi(K)$ be the infimum of $V(T, G)$ for all $G \supset K$, $G \in \mathfrak{G}_0$, and let $\varphi_r(K)$, $\varphi_r^+(K)$, $\varphi_r^-(K)$ be the analogous set functions defined in relation to $V(T_r, G)$, $V^+(T_r, G)$, $V^-(T_r, G)$, respectively. In the terminology of Halmos[3] the following results are proved by Cesari[1, 4]: (*a*) the functions φ, φ_r, φ_r^+, φ_r^-, are regular measures on $(A, \mathfrak{B}_0)$; (*b*) $\varphi_r^+ + \varphi_r^- = \varphi_r$, $r = 1, 2, 3$. Actually, Cesari's proofs[4] are only for the case where A is a compact admissible set, but the results have been extended by L. H. Turner to any admissible set. In addition, the following holds: (*c*) the measures φ_r^+, φ_r^-, are orthogonal; (*d*) the measures φ_r, φ_r^+, φ_r^- are absolutely continuous with respect to φ.

Let $\mathfrak{V}_r = \varphi_r^+ - \varphi_r^-$, $r = 1, 2, 3$. Then $\mathfrak{V}_r$ is a set function absolutely continuous with respect to φ (denoted by d_r by Cesari[4]). Therefore, Radon-Nikodym derivatives $\theta_r = d\,\mathfrak{V}_r/d\varphi$ exist as $(A, \mathfrak{B}_0)$-measurable functions.

Let $\theta(w)$ be the vector function $\theta(w) = (\theta_1, \theta_2, \theta_3)$, $w \in A$. The following hold: (*e*) for every set $K \in \mathfrak{B}_0$, we have $\bar{\varphi}(K) \leq \varphi(K) \leq \varphi_1(K) + \varphi_2(K) + \varphi_3(K)$, where $\bar{\varphi} = (\varphi_1^2 + \varphi_2^2 + \varphi_3^2)^{1/2}$, and moreover, $\varphi(K) = \sup \Sigma\, \bar{\varphi}\,(B)$, where the supremum is taken with respect to all partitions P of K into disjoint $(A, \mathfrak{B}_0)$-measurable sets B, and Σ ranges over all sets B of the partition P; (*f*) The Radon-Nikodym derivatives θ_r satisfy $\theta_1^2 + \theta_2^2 + \theta_3^2 = 1$ almost everywhere $(A, \mathfrak{B}_0, \varphi)$. With these results the following new definition of surface integral as a φ-integral becomes natural:

$$H(T, A, f) = \int_A f[p(w), \theta(w)]\, d\varphi.$$

This definition is meaningful for every surface $S = (T, A)$ of finite area and every function f which is bounded and B-measurable in $T(A) \times R$.

In addition, for f bounded and uniformly continuous on $T(A) \times R$, both J and H exist and the following holds:

THEOREM. *For every surface $S = (T, A)$ of finite area we have*

$$J(T, A, f) = H(T, A, f).$$

In other words, H gives a definition of surface integral and an integral representation of J valid for every (T, A) of finite area. By the use of the integral H, Turner has proved new theorems of lower semicontinuity without recourse to any special representation of the surface.

[1] L. Cesari, *Surface Area* (Princeton, N. J.: Princeton University Press, 1956).

[2] L. Cesari, "La nozione di integrale sopra una superficie in forma parametrica," *Ann. scuola norm. super. Pisa*, **13**, 78–117, 1948.

[3] P. Halmos, *Measure Theory* (New York: D. Van Nostrand Co., 1950).

[4] L. Cesari, "L'area di Lebesgue come una misura," *Rend. mat. appl. Roma*, **14**, 655–73, 1955.

*SPECTRAL REPRESENTATION OF SEMIGROUPS OF NORMAL OPERATORS**

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1. Let $\mathcal{G}$ be a locally compact group, $\mathcal{S}$ be a locally compact subspace of $\mathcal{G}$, and μ be the restriction to $\mathcal{S}$ of a Haar measure on $\mathcal{G}$. Let us suppose:

(1) $xy = yx \in \mathcal{S}$ for every $x, y \in \mathcal{S}$.

(2) $\mu(U) > 0$ for every subset $U \subset \mathcal{S}$, open in $\mathcal{S}$.

A complex valued function χ defined on $\mathcal{S}$ is a *character* of $\mathcal{S}$ if $\chi \not\equiv 0$, χ is continuous, and $\chi(ts) = \chi(t)\chi(s)$ for every $s, t \in \mathcal{S}$. Let us denote by E the set of characters of $\mathcal{S}$ endowed with the topology of uniform convergence on compact subsets of $\mathcal{S}$. Let $\mathfrak{U}$ be a class of open (in $\mathcal{S}$) relatively compact subsets of $\mathcal{S}$ which covers $\mathcal{S}$. For any family $\mathfrak{R} = (\mathfrak{r}(X))_{X \in \mathfrak{U}}$ of numbers ≥ 1, let $E(\mathfrak{R})$ be the subspace of characters $\chi \in E$ satisfying the inequality $|\chi(t)| \leq \mathfrak{r}(X)^{1/2}$ for $t \in X$, $X \in \mathfrak{U}$.

Proposition 1. *$E(\mathfrak{R})$ is locally compact for every family $\mathfrak{R}$.*

2. Let us denote by N_1 the set of objects $\{H, U_t\}$, where H is a Hilbert space and $t \to U_t$ a weakly continuous map of $\mathcal{S}$ into the set of bounded normal operators on H, such that $U_tU_s = U_{ts}$ for every $t, s \in \mathcal{S}$. For every family $\mathfrak{R} = (\mathfrak{r}(X))_{X \in \mathfrak{U}}$ of numbers ≥ 1, let us denote by $N_1(\mathfrak{R})$ the subset of objects $\{H, U_t\} \in N_1$ satisfying the inequality $\|U_t\| \leq \mathfrak{r}(X)^{1/2}$ for $t \in X$, $X \in \mathfrak{U}$. Any object $\{H, U_t\} \in N_1$ belongs to a subset $N_1(\mathfrak{R})$.

Theorem 1. *For every object $\{H, U_t\} \in N_1(\mathfrak{R})$ there exists one, and only one, spectral family[1] $(\mu_{x,y})_{x \in H,\, y \in H}$ on $E(\mathfrak{R})$ such that, for $t \in \mathcal{S}$ and $x, y, \in H$,*

$$(\alpha) \qquad (U_t x \mid y) = \int_{E(\mathfrak{R})} \chi(t) d\mu_{x,y}(\chi).$$

The condition, (β) $\|\mu_{x,x}\| = \|x\|^2$ for any $x \in H$, is satisfied if and only if for every nonzero $x \in H$ there is a $t \in \mathcal{S}$ such that $U_t x \neq 0$.

The formula (α)[2] can be proved by a variant of a method due to Phillips[3] and Nussbaum.[4] If $\mathcal{S} \ni e$ and $U_e = I$, the formula (α) can be deduced from theorem 5 from *FTP*[5] as follows:

Let $G = \mathcal{G} \times \mathcal{G}$, $S = \mathcal{S} \times \mathcal{S}$, $m = \mu \otimes \mu$ and $x^+ = (t, s)^+ = (s, t)$ for all $x = (t, s) \in S$. Then $\{G, S, m, x \to x^+\}$ has the properties 1–4 formulated in *FTP*. Let $\mathfrak{V} = (X \times Y)_{X \in \mathfrak{U},\, Y \in \mathfrak{U}}$ and $\mathfrak{R} \otimes \mathfrak{R} = (\mathfrak{r}(X)\mathfrak{r}(Y))_{X \times Y \in \mathfrak{V}}$. Then $\mathfrak{g}: \chi \to \chi'$, where χ' is defined on S by the equality $\chi'(x) = \chi'((t, s)) = \chi(t)\overline{\chi}(s)$, is a homeomorphism of $E(\mathfrak{R})$ onto $A(\mathfrak{R} \otimes \mathfrak{R})$. If we define $V_{(t,s)} = U_tU_s^*$ for $(t, s) \in S$, then $\{H, V_{(t,s)}\} \in R(\mathfrak{R} \otimes \mathfrak{R})$, and hence there exists a spectral family $(\nu_{x,y})_{x \in H,\, y \in H}$ on $A(\mathfrak{R} \otimes \mathfrak{R})$ such that $(U_tU_s^*x \mid y) = \int_{A(\mathfrak{R} \times \mathfrak{R})} \chi'((t, s)) d\nu_{x,y}(\chi')$ for $(t, s) \in S$ and $x, y \in H$. If $\mu_{x,y}(f) = \nu_{x,y}(f \circ g^{-1})$ for $x, y \in H$ and $f \in \mathfrak{K}(E(\mathfrak{R}))$, then the family $(\mu_{x,y})_{x \in H,\, y \in H}$ is spectral and verifies the equality (α) for $t \in \mathcal{S}$ and $x, y \in H$.

Remarks.—(*a*). If P is the projection valued measure corresponding to the spectral family $(\mu_{x,y})_{x \in H, y \in H}$, then the condition (β) is equivalent to the equality $P(E(\mathfrak{R})) = I$.

(*b*) Let $t \to t^+$ be a continuous representation of $\mathcal{S}$ into $\mathcal{S}$ such that: (i)$t^{++} = t$ for any $t \in \mathcal{S}$; (ii) T^+ is locally negligible if T is locally negligible. Let us denote by A the subspace of characters $\chi \in E$ satisfying the equality $\chi(t^+) = \overline{\chi(t)}$ for $t \in \mathcal{S}$,

and, for every family $\mathfrak{N} = (\mathfrak{r}(X))_{X \in \mathfrak{U}}$ of numbers ≥ 1, let $A(\mathfrak{N}) = E(\mathfrak{N}) \cap A$. If the object $\{H, U_t\}$ in Theorem 1 verifies the equality $U_{t^+} = U_t^*$ for every $t \in \mathcal{S}$, then the carrier of every measure $\mu_{x,y}$ is contained in $A(\mathfrak{N})$.

(*c*) The preceding results contain, in particular, some classical theorems by Hille,[5] Stone,[7] and Nagy.[8]

3. The following result gives a criterion of local compactness of E:

PROPOSITION 2. *E is locally compact if there exists a compact subset $V \subset \mathcal{S}$, having the property: for every $t \in \mathcal{S}$ there is an integer $p > 0$ such that $t^p \in \bigcup_{j=1}^{\infty} i(V^j)$*[9].

Let us suppose, now, that three more conditions are satisfied:

(3) The interior of $\mathcal{S}$ is nonvoid.

(4) There is a denumerable subset of $\mathcal{S}$ dense in $\mathcal{S}$.

(5) E is locally compact.

If $\mathcal{G} = Z^n \times R^m$ (n, m are integers > 0, Z is the additive group of the integers, R is the additive group of the real numbers) and $\mathcal{S}$ is the subset of systems $(x_1, \ldots, x_{n+m}) \in \mathcal{G}$ such that $x_1 \geq 0, \ldots, x_{n+m} \geq 0$, $x_1 + \ldots + x_{n+m} \neq 0$, then conditions (1)–(5) are satisfied.

Let us denote by N_2 the set of objects $\{H, U_t\}$ where H is a Hilbert space and $t \to U_t$ a map of $\mathcal{S}$ into the set of normal operators on H, not necessarily bounded, such that:

(i) $U_t U_s = U_{ts}$ for $t, s \in \mathcal{S}$;

(ii) $t \to (U_t x \mid y)$ is continuous for $x \in D = \bigcap_{t \in \mathcal{S}} D(U_t)$[10] and $y \in H$.

THEOREM 2. *For every object $\{H, U_t\} \in N_2$ there exists one, and only one, spectral family $(\mu_{x,y})_{x \in H, y \in H}$ on E, such that, for every $t \in \mathcal{S}$, $x \in D(U_t)$, and $y \in H$,*

$$(U_t x \mid y) = \int_E \chi(t) d\mu_{x,y}(\chi).$$

The condition (β) is verified if and only if for every nonzero $x \in D$ there is a $t \in S$ such that $U_t x \neq 0$.

Remarks.—(*a*) If $U_{t^+} = U_t^*$ for any $t \in \mathcal{S}$, where $t \to t^+$ is as in remark (*b*) above, then the carrier of every measure $\mu_{x,y}$ is contained in A.

(*b*) The representation formulas given by Devinatz (*Proc. Am. Math. Soc.*, **5,** 101–2, 1954), Getoor (*Proc. Am. Math. Soc.*, **7,** 387–91, 1956), and Devinatz and Nussbaum[2] are particular cases of the preceding results.

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[1] A family $(\mu_{x,y})_{x \in H, y \in H}$ of Radon measures, on a locally compact space B, is spectral if: (1) $x \to \mu_{x,y}$ is linear for every $y \in H$; (2) $\mu_{x,y} = \bar{\mu}_{y,x}$ for every $x, y \in H$; (3) $\|\mu_{x,y}\| \leq \|x\| \, \|y\|$ for every $x, y \in H$; (4) $g \cdot \mu_{x,y} = \mu_{U_g x, y}$ for every $g \in \mathcal{K}(B)$ and $x, y \in H$, where U_g is the operator on H defined by the equalities $(U_g x \mid y) = \int_B g(\chi) d\mu_{x,y}(\chi)$, $x, y \in H$.

[2] See also A. Devinatz and A. E. Nussbaum, *Ann. Math.*, **65,** 144–52, 1957.

[3] *Trans. Am. Math. Soc.*, **71,** 393–415, 1951.

[4] *Duke Math. J.*, **22,** 573–82, 1955.

[5] We design by *FTP* the note: "Fonctions de type positif," *Compt. rend. Acad. Sci. Paris*, **243,** 1389–92, 1956. We shall use the notations introduced in this note without further explanations. Let us remark that the condition (*) from *FTP* can always be supposed verified.

[6] These PROCEEDINGS, **24,** 159–61, 1938.

[7] *Ann. Math.*, **33,** 643–48, 1932.

[8] *Spektraldarstellung linearer Transformationen in Hilbertschen Raumes* (Berlin, 1942).

[9] For a subset $X \subset \mathcal{S}$, $i(X)$ is the interior of X in $\mathcal{S}$.

[10] For any operator T we design its domain by $D(T)$.

*THE SPECTRAL LUMINOSITY CURVES FOR A DICHROMATIC EYE AND A NORMAL EYE IN THE SAME PERSON**

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Communicated November 6, 1957

We have recently had the opportunity to determine some visual functions of a unilaterally color-blind person, that is, one who demonstrates some form of color blindness in one eye and normal (trichromatic) vision in the other.

The fact that a condition of unilateral color blindness can exist has been known since 1879.[1] Cases of unilateral color blindness have been historically important, for it has been supposed that they could provide conclusive evidence on the chromatic perceptions of color-blind individuals. It is for this reason that most of the tests made upon them were concerned with comparisons between the colors seen in the normal and the color-blind eye. The evidence seems to show that in unilaterally color-blind subjects, any form of color deficit may be represented in the color-blind eye, e.g., dichromatism, anomalous trichromatism, or completely monochromatic vision. Judd[2] reports that thirty-seven cases of unilateral color blindness have been described in the literature. Because of inadequate procedure or background information, the data on only eight have proved of more than passing interest.

No case of unilateral color blindness so far reported has involved a color-blind eye whose discriminative functions are known in detail, and it is probable that in most of the cases reported the trichromatic eye has not met completely rigorous criteria of normality. We do not believe that these latter shortcomings apply in the case of our subject. We have made analyses of several of the visual functions exhibited by both the color-blind and the normal (trichromatic) eyes, and we are confident that the trichromatic eye of our subject does, in fact, meet the usual standards of normality.

A recent paper[3] by us has provided information on luminosity losses of dichromats. In that paper we pointed out the contradictory issues raised by the facts of luminosity losses in dichromats and the color-naming of unilaterally color-blind subjects. We have examined various visual functions of our unilaterally color-blind subject over a period of about three years, with considerable periods of interruption. (Since a later report on color mixture will show that, in her color-blind eye, she can match any spectral wave length by a mixture of 460 mμ and 650 mμ, we shall refer to her color blindness as a form of dichromatism.) The present paper reports the first of these studies, a description of this subject's luminosity curve for the normal and dichromatic eye. It is found that our subject, who gives typical dichromatic responses (of a predominantly deuteranopic type) on the screening tests, evidences a considerable degree of luminosity loss in the green and blue regions of the spectrum. In this respect her data are in accord with our recently published data on luminosity losses in protanopes and deuteranopes.[3]

Procedure.—The procedure used for observations with each eye of our unilaterally color-blind subject was similar to the one followed with the normal and color-blind subjects, as previously reported. Spectral lights, each of a narrow wave-

length band, were presented by means of our double monochromator. The duration of each flash, seen by the subject through an artificial pupil of 2.4 mm. in diameter, was 4 milliseconds. The diameter of the circular stimulus (which appeared within a dimly illuminated fixation ring, 2.5° in diameter) was 42 minutes. A correction of −2 diopters was required for the right eye and −4 for the left. (During the experiment the correction lenses were installed just behind the artificial pupil, on the side away from the subject.) The method of limits was used to determine threshold, and a 10-minute period of dark adaptation was given before each sequence of threshold determinations in a given experimental period. About four to six thresholds were obtainable in the usual experimental period of 1 hour.

Our subject took a considerable number of tests, including those given in the routine screening of subjects. The routine tests[4] involved (*a*) the reading of the Ishihara and Stilling plates, (*b*) the determination of the proportion of red and green required to match a yellow on the Hecht-Shlaer anomaloscope, and (*c*) the determination of the subject's neutral point made with a modified Helmholtz color-mixer.[5] In addition to the routine tests, the following were also employed: the Dvorine test (2d ed.), American Optical Company plates, Farnsworth Dichotomous D-15, and Farnsworth-Munsell 100-Hue tests.[4]

Dr. Gertrude Rand and Miss Catherine Rittler, of the laboratory of Ophthalmology, Columbia University College of Physicians and Surgeons, made additional examinations of our subject. They used the following tests for both the color-blind and the normal eye: the Hardy-Rand-Rittler pseudoisochromatic plates, the two Farnsworth tests, the American Optical Company plates, the Ishihara test, the Rabkin test, and the Boström-Engelberg test.[4] The Nagel anomaloscope[5] was used on both eyes. Dr. Rand and Miss Rittler concluded that, although our subject's right eye is normal by all the tests used, including the Nagel anomaloscope, her left eye does not fit a category of classical color defect. We deeply appreciate the co-operation of Dr. Rand and Miss Rittler. We are also indebted to Dr. R. L. Pfeiffer, of the Ophthalmological Institute, Columbia University College of Physicians and Surgeons, for the ophthalmological examination of our subject. He found no organic disease.

The Luminosity Curve.—The luminosity curves of our subject are given in Figure 1. The figure shows that in the red end of the spectrum both eyes of our subject have about the same degree of sensitivity. The curves for the two eyes differ in absolute luminosity values in the blue and green regions of the spectrum.[6] The luminosity of the normal eye in those regions is comparable to the luminosity given by normal subjects, but the luminosity of the color-blind eye shows considerable loss as contrasted with the normal eye. The data for the color-blind eye look like the data for a deuteranope, although it might be said that the luminosity loss occurs at wave lengths probably unusually far into the orange. In any case, the subject shows a considerable degree of luminosity loss in the green and blue for her color-blind eye.

Discussion.—Our unilaterally dichromatic subject exhibits a considerable degree of luminosity loss in the color-blind eye, particularly in the orange to blue region of the spectrum. In this respect she shows an effect comparable to that shown by five of six deuteranopes that we have tested.[3]

No claim is made, despite the comparability of our subject's data to those found

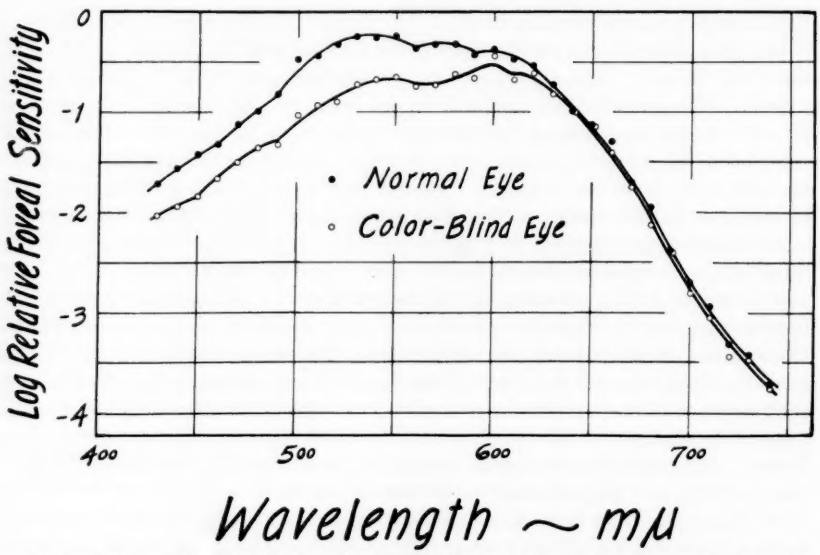

FIG. 1.—Log foveal sensitivity values for threshold in the trichromatic and color-blind eye of a unilaterally color-blind subject.

earlier with deuteranopes,[3] that she is, in fact, a simple deuteranope. Later reports will show, as did the diagnostic testing of Dr. Rand and Miss Rittler, that our subject may not be typically deuteranopic. For example, her hue discrimination is better than that of the usual deuteranope in the short-wave region of the spectrum.

Summary.—We have determined the respective luminosity curves of the two eyes of a unilaterally color-blind person. The subject's right eye is normal, and her dichromatic eye, except for a few details, is most directly classifiable as deuteranopic. Her color-blind eye shows a loss of luminosity as contrasted with her normal eye in the spectral range from about 625 mμ to 430 mμ. The loss that she exhibits is greater than comparable ones shown by five out of six deuteranopes in a previous report.[3]

* This work was supported by a contract between the Office of Naval Research and Columbia University and by a grant-in-aid from the Higgins Fund of Columbia University. Reproduction in whole or in part is permitted for any purpose of the United States government. An abstract of some of the present research has appeared earlier (see C. H. Graham and Yun Hsia, *Science*, **120**, 780, 1954). We wish here to express our deep appreciation to our subject (A. H. C.) for the many hours of observation that she devoted to our work.

[1] The four following references are the earliest, the second, third, and fourth being concerned with the famous case of Hermann Goldenberg, the nature of whose color blindness still remains in controversy: O. Becker, *Graefe's Arch. Ophthalmol.*, **25**, 205–212, 1879; A. Von Hippel, *Graefe's Arch. Ophthalmol.*, **26**, 176–186, 1880; F. Holmgren, *Proc. Roy. Soc. London*, **31**, 302–306, 1881; A. Von Hippel, *Graefe's Arch. Ophthalmol.*, **27**, 47–55, 1881.

[2] For a history and analysis of the problem see D. B. Judd, *J. Research Nat. Bur. Standards*, **41**, 247–271, 1948. The latest research on unilateral color blindness reported by Judd is that of Louise L. Sloan and Lorraine Wollach, *J. Opt. Soc. Amer.*, **43**, 890–894, 1953.

[3] Y. Hsia and C. H. Graham, these PROCEEDINGS, **43**, 1011, 1957.

[4] On the tests used, see the following. S. Ishihara, *Tests for Colour-Blindness* (6th ed.; Tokyo:

Kanehara, 1932); E. Hertel, *Stilling's Pseudo-isochromatische Tafeln* (Leipzig: Georg Thieme, 1936); I. Dvorine, *Dvorine Pseudo-isochromatic Plates* (2d ed.; Baltimore: Waverly Press, 1953); American Optical Company, *A-O Pseudo-isochromatic Plates* (Philadelphia: Beck Engraving, 1940); D. Farnsworth, *Farnsworth-Munsell 100-Hue Test* (Baltimore: Munsell Color Co., 1949); D. Farnsworth, *The Farnsworth Dichotomous Test for Color Blindness* (New York: Psychological Corporation, 1947); L. H. Hardy, G. Rand, and M. C. Rittler, *H-R-R Pseudoisochromatic Plates* (1st ed.; American Optical Co., 1955); E. Rabkin, *Polychromatic Plates for Testing Color Vision* (2d ed.; Kiev: State Medical Publishing House, 1939); C. D. Boström and I. Kugelberg, *Plates for Testing Color Vision*, Vol. I (Stockholm: Kifa, 1943).

[5] On the Helmholtz color-mixer see S. Hecht and S. Shlaer, *J. Gen. Physiol.*, **20**, 57, 1936. On the Hecht-Shlaer and also the Nagel anomaloscope see M. P. Willis and D. Farnsworth, *Med. Research Lab., U.S. Submarine Base*, Vol. **11**, No. 7, Rept. No. 190, 1952.

[6] Log_{10} energy values for threshold in the two eyes of our subject. [Log threshold values relative to the average log threshold energy values for 7 normal subjects (in reference of footnote 3) with unit determined by setting log energy = 0 at 545 $m\mu$, the maximum of the luminosity curve. Absolute energy for average threshold of the normal subjects is (interpolated) 3.5×10^{-8} erg (about 10,000 quanta) at 578 $m\mu$.] Log_{10} energy values for the trichromatic (normal) eye of our unilaterally color-blind subject at steps of 10 $m\mu$ beginning with 430 $m\mu$ and ending with 740 $m\mu$ are as follows: 1.71, 1.57, 1.42, 1.33, 1.13, 1.01, 0.82, 0.48, 0.45, 0.33, 0.26, 0.27, 0.25, 0.37, 0.33, 0.33, 0.44, 0.38, 0.48, 0.54, 0.73, 1.00, 1.13, 1.30, 1.71, 1.95, 2.38, 2.71, 3.01, 3.32, 3.42, 3.72. Comparable data for the dichromatic (left) eye for the same spectral range are as follows: 2.04, 1.95, 1.86, 1.67, 1.50, 1.36, 1.34, 1.05, 0.94, 0.91, 0.74, 0.79, 0.67, 0.75, 0.75, 0.63, 0.67, 0.44, 0.69, 0.62, 0.87, 0.99, 1.14, 1.42, 1.76, 2.13, 2.40, 2.82, 3.05, 3.46, 3.48, 3.74.

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